DEVELOPMENT OF VALIDATED ANALYTICAL METHODS FOR THE SIMULTANEOUS DETERMINATION OF METFORMIN AND TENELIGLIPTIN FROM TABLET DOSAGE FORM

A Dissertation submitted to

THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY,

CHENNAI – 600 032

In partial fulfilment of the requirements for the award of the Degree of

MASTER OF PHARMACY

IN

BRANCH–V- PHARMACEUTICAL ANALYSIS

Submitted by

R. ARAVIND RAJ
REGISTRATION No. 261530101

Under the guidance of

Dr. SUSHEEL JOHN VARGHESE, M.Pharm., Ph.D.
Department of Pharmaceutical Analysis

COLLEGE OF PHARMACY
SRI RAMAKRISHNA INSTITUTE OF PARAMEDICAL SCIENCES
Coimbatore - 641 044.

APRIL 2017

CERTIFICATE

This is to certify that the dissertation entitled **"Development of Validated Analytical Methods for the Simultaneous Determination of Metformin and Teneligliptin from Tablet Dosage Form"** being submitted to The Tamil Nadu Dr.M.G.R Medical University, Chennai was carried out by **R. Aravind Raj** in the **Department of Pharmaceutical Analysis,** College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, under the supervision and guidance of **Dr. Susheel John Varghese, M.Pharm., Ph.D.** Assistant Professor, Department of Pharmaceutical Analysis, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore.

Dr. T. K. RAVI, M. Pharm., Ph.D., FAGE.,
Principal & HOD,
Department of Pharmaceutical Analysis,
College of Pharmacy, SRIPMS,
Coimbatore-641 044.

Place: Coimbatore

Date:

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Dr. SUSHEEL JOHN VARGHESE, M.Pharm., Ph.D.
Assistant Professor,
Department of Pharmaceutical Analysis,
College of Pharmacy, SRIPMS,
Coimbatore-641 044.

Place: Coimbatore

Date:

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ABBREVIATIONS AND SYMBOLS

HPLC	-	High Performance Liquid Chromatography
HPTLC	-	High Performance Thin Layer Chromatography
ICH	-	International conference on harmonization
LOD	-	Limit of detection
LOQ	-	Limit of quantification
min	-	Minute
RP-HPLC	-	Reverse Phase - High Performance Liquid Chromatography
RSD	-	Relative standard deviation
UV	-	Ultra violet
A_S	-	Asymmetric factor
gm	-	Gram
M	-	Molar
Mg	-	Milligram
ml	-	Milliliter
mM	-	Millimolar
mm	-	Millimeter
N	-	Normality
µg	-	Microgram
ng	-	Nano gram
R_f	-	Retardation factor

Rs	-	Resolution
Rt	-	Retention time
T_f	-	Tailing factor
k`	-	capacity factor
α	-	selectivity
µl	-	Microlitre
λmax	-	Wavelength of maximum absorbance
MET	-	Metformin
TENE	-	Teneligliptin
WHO	-	World Health Organisation

CONTENTS

1. INTRODUCTION

Analytical chemistry deals with methods for determining the chemical composition of samples of matter. Analytical chemistry consists of classical, wet chemical methods and modern, instrumental methods. Analytical chemistry has broad applications to forensics, medicine, science and engineering.

I. CLASSICAL METHODS[1]

1. Qualitative methods
2. Quantitative methods

1. Qualitative methods

A qualitative method yields information about the identity of atomic or molecular species or the functional groups in the sample. Identification may be based on differences in colour, odour, melting point, boiling point, radioactivity or reactivity.

2. Quantitative methods

A quantitative method provides numerical information as to the relative amount of one or more of these components. It uses mass or volume changes to quantify amount.

II. INSTRUMENTAL METHODS

The instruments used for the analysis of methods are given below:

- X – ray diffraction
- UV – Visible Spectroscopy
- Raman spectroscopy
- Refractrometry
- Polarimetry
- Potentiometry

- ❖ Polarography
- ❖ Conductometry
- ❖ Mass spectrometry

There are several valid reasons for developing new method of analysis:

- ➢ Marketed drug may not be official in pharmacopoeias.
- ➢ There may not be a suitable method for a particular analyte in the specific sample matrix.
- ➢ Existing methods may have poor accuracy and precision, too expensive, time consuming or energy intensive, not easily automated, does not provide adequate sensitivity or analyte selectivity in sample of interest.
- ➢ Difficulties in extraction and separation process.
- ➢ There may be a need for an alternative method to confirm, for legal or scientific reasons, analytical data originally obtained by existing methods.

III. CHROMATOGRAPHY[2]

Chromatography is essentially a group of techniques for the separation of the compounds of mixtures by their continuous distribution in two phases, one of which moving past the other. The systems associated with this definition are

- ❖ A solid stationary phase and a liquid or gaseous mobile phase (adsorption chromatography)
- ❖ A liquid stationary phase and a liquid or gaseous mobile phase (partition chromatography)
- ❖ A solid polymeric stationary phase containing replaceable ions and an ionic liquid mobile phase (ion exchange chromatography)
- ❖ An inert gel which acts as a molecular sieve and a liquid mobile phase (gel chromatography)

The basis of the separation of the components of a mixture may be defined in terms of one of these four modes of separation or by a combination.

1. CHROMATOGRAPHY TECHNIQUES

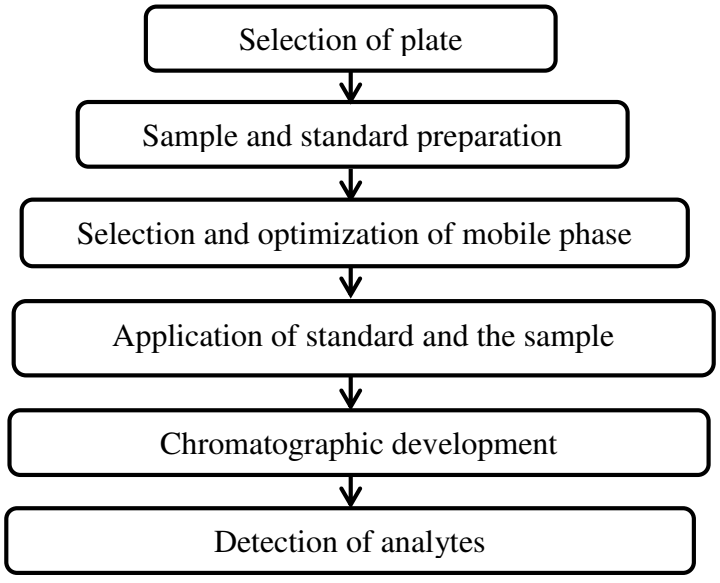
The different chromatographic techniques include:

- ❖ Thin layer chromatography
- ❖ High performance thin layer chromatography
- ❖ High performance liquid chromatography
- ❖ Gas chromatography
- ❖ Super critical fluid chromatography

1.1. High Performance Thin Layer Chromatography[3, 4]

HPTLC is a sophisticated and automated form of TLC and is highly useful method for both qualitative and quantitative analysis. It allows for the various analytical applications which involve complex separation. The advantages of automation, scanning, full optimization, selective detection principle, minimum sample preparation, hyphenation, etc. enable it to be a powerful analytical tool for chromatographic information of complex mixtures of inorganic, organic, and biomolecules. It is a valuable tool for reliable identification providing chromatographic fingerprints.

Various steps involved in HPTLC



a. Selection of plate

Pre-coated plates with various support material, sorbent layer and with various sorbent thickness of 100-250 μm are used for quantitative and qualitative analysis.

b. Sample preparation

Sample preparation involves, dissolving the dosage form in a solvent with complete recovery of intact compounds of interest and minimum of matrix with suitable concentration of analyte during extraction and analysis must be considered and ensured.

Choice of solvent for the sample:

- It should dissolve the analytes.
- It should be reasonably volatile.
- It should have low viscosity.
- It should be a weak (least polar) chromatographic solvent for the analyte.

c. Sample application

This is the most critical step for obtaining good resolution for quantification by HPTLC. The sample solution should be applied through clean upper end of the capillary to marked point. Usually application of 0.5-5μl for HPTLC is recommended. Samples are applied as bands, because it offers better separation and uniform after development.

Advantages of auto sampler:

- Even distribution of sample
- Better resolution
- Greater accuracy
- Better separation
- Larger quantities of sample can be handled for application

d. Optimization of mobile phase

Mobile phase should be chosen by considering chemical properties of analytes and the sorbent layer. Mobile phase ratio is selected by trial and error method.

e. Chromatographic development

The chamber needs to be saturated prior to development, as solvent vaporizes soon. The time required for saturation will depend on the nature and composition of mobile phase and layer thickness. The precoated plate is then placed in the saturated chamber containing mobile phase and allowed to run the desired running distance and then kept out for drying.

f. Detection

- Detection under UV light is the first choice, as it is non – destructive in most cases and employed for densitometric scanning.
- Iodine is the universal detection reagent, the detection is usually non-destructive and reversible but certain substances may be altered through non-reversible derivatization such as ethambutol hydrochloride, a totally non-UV absorbing compound.
- Fluorescent chemicals are employed for detection of lipophilic substances by wetting/non-wetting technique.

1.2. High Performance Liquid Chromatography[5-7]

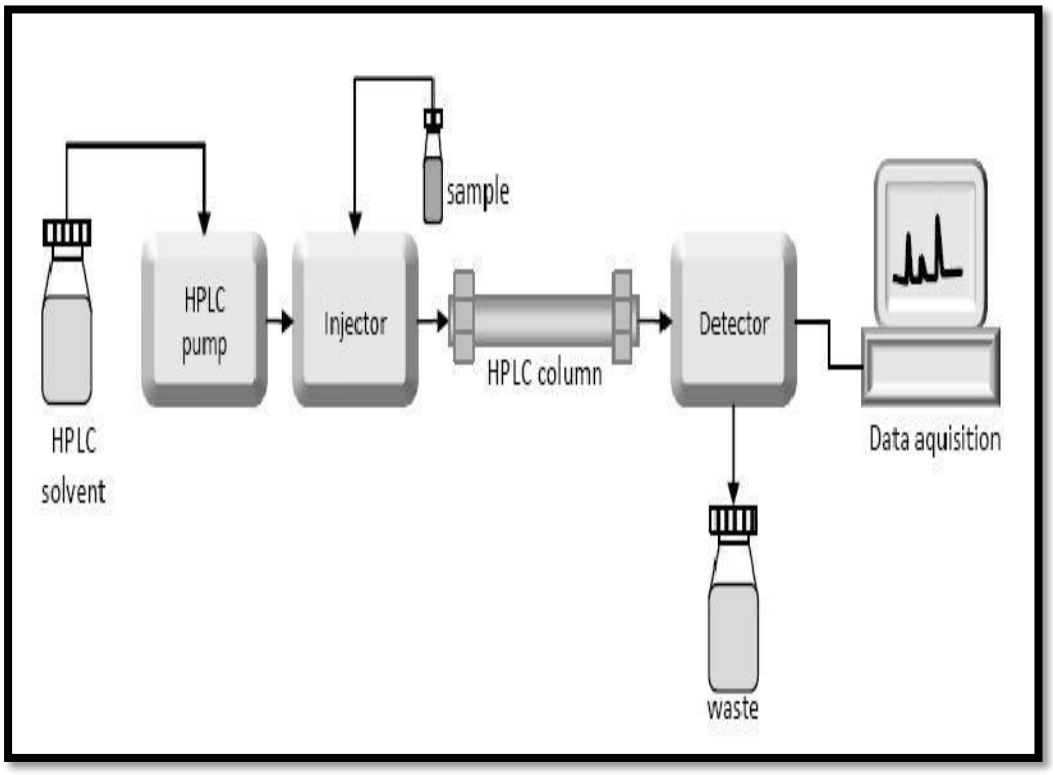
A variety of methods are available for analysing pharmaceutical compounds; however high pressure liquid chromatography is currently the method of choice for the analysis of these compounds. HPLC offers high performance over ambient pressure or low pressure liquid chromatography.

High performance liquid chromatography is used in analytical development to quantitate the active pharmaceutical ingredient (API) and to evaluate impurity and degradation product profiles of drug substance and drug products. Additional uses of HPLC include the determination of content uniformity of dosage forms, monitoring of dissolution profiles, determination of antioxidant and microbial preservative content and support of cleaning validations. Separations of these types require only a monitoring of one, or a limited number of predefined components. A significantly larger challenge is presented in the composite assays of drug substances and drug products where the goal is to quantitate API and relevant impurities and degradation products in a single chromatographic run.

The method development of pharmaceuticals by HPLC begins with array of methods suitable for the separation of drug substances, synthetic intermediates and starting materials, excipients, and products from forced decomposition studies.

The development of any new or improved method usually tailors existing approaches and instrumentation to the current analyte, as well as to the final needs or requirements of the method. Method development usually requires selecting the requirements and deciding on what type of instrumentation to utilize at the development stage, decisions regarding

- Choice of column,
- Mobile phase,
- Detector(s) and
- Method of quantitation must be addressed



Goals for new or improved HPLC method might include the following:

- Qualitative identification of the specific analyte(s) of interest, providing some structural information to confirm general behaviour (i.e. retention time, pH)
- Quantitative determination, at trace levels when necessary (i.e. accurate, precise and reproducible in any laboratory setting when performed according to established procedures).
- Ease of use, ability to be automated, high sample throughout and rapid sample turnaround time.
- Sample preparation that minimizes time, effort, materials and volume of sample consumed.

The following criteria are to be met for developing methods:

i. For drug substance

Methods should separate the API, synthetic process impurities, and drug substances degradation products. Methods should be able to detect impurities and degradation products present at levels greater than 0.05% relative to the API. Impurities and degradation products present at the levels greater than 0.1% should be identified and specifications should be placed on limits.

ii. For drug products

Methods should separate the API, drug products degradation products from excipients. Drug products methods are not required to monitor synthetic process impurities, unless they are also drug product degradation products. Methods should be able to detect degradation products present at levels greater than 0.1% relative to the API. Degradation products present at levels greater than 0.2% should be identified and specifications should be placed on limits.

1.2.1. Fundamental Concepts

The fundamental concepts in HPLC include:

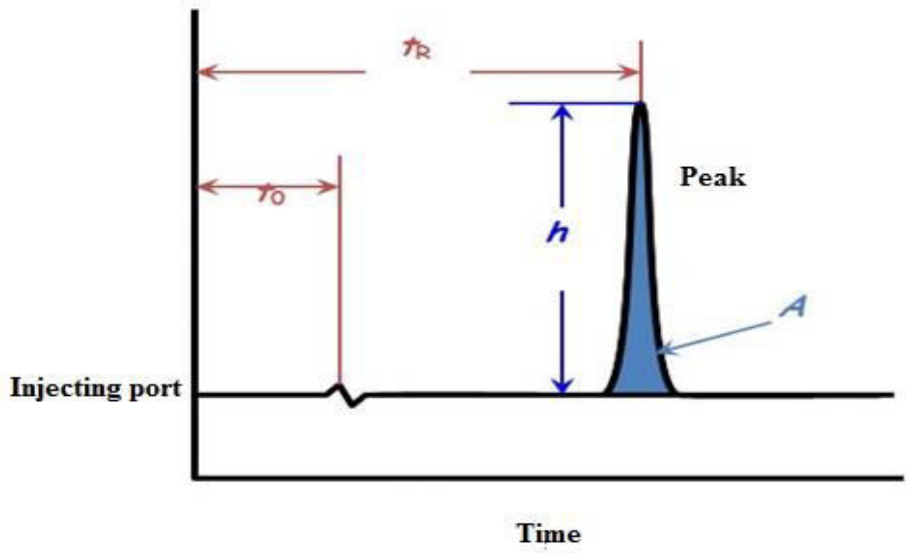
a. Retention time

Retention time is the time of emergence of the peak maximum of a component after injection.

The time between the sample injection point and analyte reaching a detector is called retention time (t_R). The retention time of an unretained component (often marked by the first baseline disturbance caused by the elution of the sample solvent) is termed void time (t_0).

The height or the area of a peak is proportional to the concentration or the amount of that particular component in the sample. The peak area is most commonly used since it provides a more accurate quantitative measurement.

Chromatogram



b. Capacity factor (k`)

A more fundamental term that measures the degree of retention of the analyte is the capacity factor or retention factor (k`), calculated by normalising the net retention time by the void time (t_0).

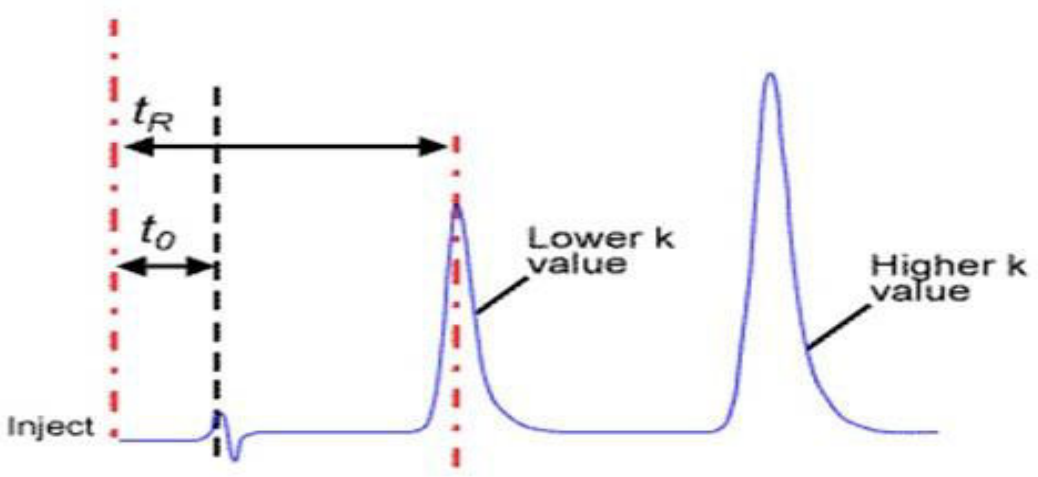
$$k` = t_R - t_0 \ / \ t_0$$

Where

k` = capacity factor

t_R = retention time

t_0 = void time



c. Selectivity (α)

Separations between two components are possible if they have different migration rates through the column. Selectivity or separation factor is a measure of differential retention of two analytes.

Selectivity must be greater than 1.5 for peak separation. Selectivity is dependent on the nature of the stationary phase and the mobile phase composition.

d. Column efficiency (N)

The term plate number (N), is a quantitative measure of the efficiency of the column and is related to the ratio of the retention time and the standard deviation of the peak width (σ).

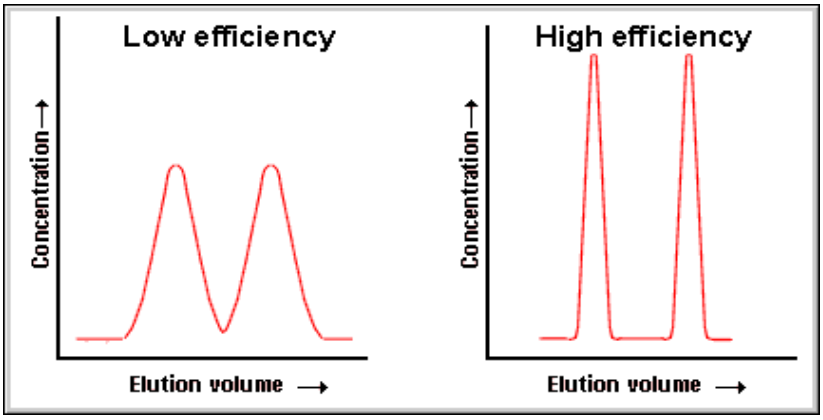
$$N = 16 \text{ X } (t_R / W)$$

Where

N = number of theoretical plates

t_R = retention time

w = width of the peak



e. Height equivalent of a theoretical plate (HETP)

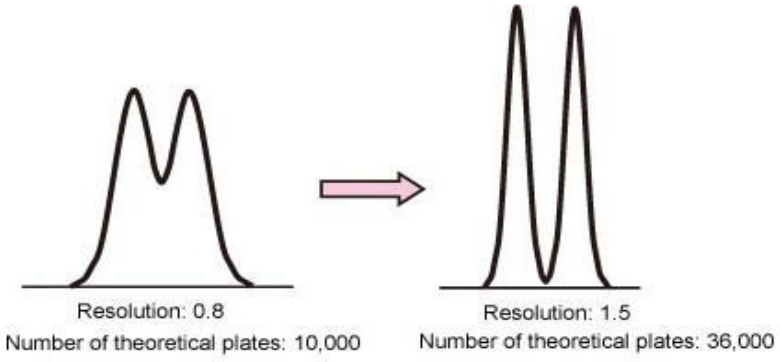
The concept of a plate is traditionally derived from the industrial distillation process using distillation columns consisting of individual plates were the condensing liquid is in equilibrium with the rising vapour. Thus, a longer distillation column would have more plates or equilibration steps. Similarly in chromatography, the HETP is equal to the length of the column (L) divided by the number of theoretical plates (N) even though there are no discrete plates inside the HPLC column.

Height equivalent of a theoretical plate, (HETP) = L / N

Where

L = length of the column

N = number of theoretical plates



f. Resolution (R_s)

The goal of most HPLC analysis is the separation of one or more analytes from other components in the sample in order to obtain quantitative information for each analyte. Resolution is the degree of separation of two adjacent peaks, and is defined as the difference in retention times of the two peaks divided by the average peak width. As peak widths of adjacent peaks tend to be similar, the average peak width can be equal to the width of one of the two peaks.

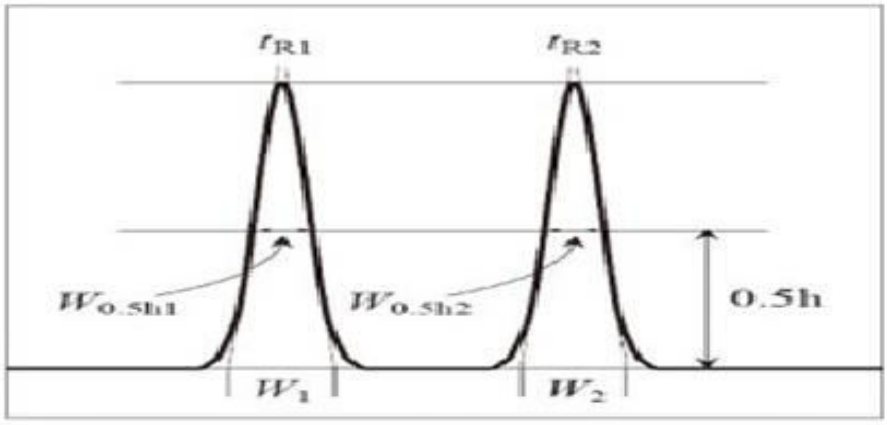
$$\text{Resolution, } (R_s) = t_{R1} - t_{R2} / \{(w_{1+} w_2)/2\}$$

Where

t_{R1} & t_{R2} - retention time of peaks

w_1& w_2 - width of the peaks

Resolution: Chromatogram with two adjacent peaks

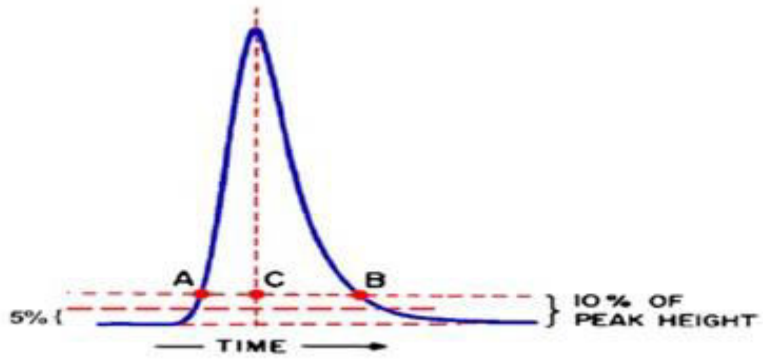


g. Tailing factor (T_f)

Under ideal conditions, chromatographic peaks will have Gaussian peak shapes with perfect symmetry. In reality, most peaks are either slightly fronting or tailing. The tailing factor is defined by the USP, as a measure of peak asymmetry.

Tailing factors for most peaks should fall between 0.9 and 1.4, with a value of 1.0 indicating a perfectly symmetrical peak. Peak tailing is typically caused by adsorption or other strong interactions of the analyte with the stationary phase while peak fronting can be caused by column overloading, chemical reaction or isomerization during the chromatographic process.

Tailing factor, $T_f = AC / 2AB$



1.2.2. Mobile Phase Parameters

a. Organic solvent and selectivity

Sample retention can be controlled by varying the solvent strength of the mobile phase. A strong solvent decreases retention and weak solvent increases retention. Tetrahydrofuran is stronger than acetonitrile, which in turn is stronger than methanol in RP – HPLC.

b. Buffers

Many drugs have either acidic or basic functional groups and can exist in solutions in ionized or non – ionized forms. The ionic state and degree of ionization greatly affect their chromatographic retention in RP – HPLC. Typically, the ionic form does not partition well into the hydrophobic stationary phase and therefore has significantly lower capacity factor than the neutral, un-ionized form. Buffers are commonly used to control the pH of the mobile phase for the separation of acidic or basic analytes.

c. Acidic mobile phase

A mobile phase at acidic pH of 2.5 – 3 is a good starting point for most pharmaceutical applications because the low pH suppresses the ionization of most acidic analytes resulting in the higher retention. Common acids used for mobile phase preparations are phosphoric acid, formic acid and acetic acid. Low pH also minimises the interaction of basic analytes with surface silanols on the silica packing (because silanols do not ionize at acidic pH). Also, the lifetime of most silica-based columns is excellent in the pH range of 2–8. However, basic analytes are ionized at low pH and might not be retained unless ion – pairing reagents are used.

d. Ion – pairing reagents

Ion – pairing reagents are detergents like molecules added to the mobile phase to provide additional retention or selectivity for the analytes with opposite charge. Long- alkyl sulphonates are commonly used for the separation of water soluble basic analytes in the analysis of water soluble vitamins. Amine modifiers such as trimethylamine are often used in the mobile phase to reduce peak tailing caused by strong interaction of basic analytes with acidic surface silanols. For acidic analytes, ion pairing reagents such as tetra – alkyl ammonium salts are often used.

e. Isocratic vs gradient analysis

Traditionally, most pharmaceutical assays are isocratic analysis employing the same mobile phases throughout the elution of the sample. Isocratic analyses are particularly common in quality control applications since they use simpler HPLC equipment and premixed mobile phases.

In contrast, gradient analysis in which the strength of the mobile phase is increased with time during sample elution, is suited for complex samples and those containing analytes of wide polarities. Gradient chromatography is amenable for high – through put screening applications and for impurity testing. It yields better seperation for early peaks and sharper peaks for late eluters.

IV. VALIDATION PARAMETERS AS PER ICH GUIDELINES[8]

a. Specificity

Specificity is the ability to assess unequivocally the analyte in presence of components which may be expected to be present like impurities, matrix etc. An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities and the assay. The procedures used to demonstrate specificity will depend on the intended objective of the analytical procedure.

b. Accuracy

The accuracy of an analytical procedure expresses the **c**loseness of agreement between the value which is accepted either as convential true value or an accepted reference value and the value found. Accuracy should be established across the specified range of the analytical procedure.

- Assay
  1) Drug substance
  2) Drug product
- Impurities (Quantitation)
- Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations/3 replicates each of the total analytical procedure). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

c. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Acceptance criteria: %RSD < 2

i. Repeatability (Intra assay precision**)**

Repeatability expresses the precision under the same operating conditions over a short interval of time.

ii. Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

iii. Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

d. Limit of detection (LOD)

LOD is defined as the smallest concentration of an analyte detected, but not necessarily quantified. A few approaches to determine LOD are listed below

i. Based on signal-to-noise approach

A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

ii. Based on the standard deviation of the response and the slope

The detection limit (DL) may be expressed as:

$$DL = 3.3\ \sigma / S$$

Where

σ = the standard deviation of the response

S = the slope of the calibration curve

e. Limit of quantification (LOQ)

LOQ is defined as the lowest concentration of an analyte determined with acceptable precision, accuracy and reliability by a given method under stated experimental conditions. A few approaches to determine LOQ are listed below

i. Based on signal-to-noise approach

A signal-to-noise ratio of 10: 1 is generally considered acceptable for estimating the quantitation limit.

ii. Based on the standard deviation of the response and the slope

The quantitation limit (QL) may be expressed as:

$$\text{Quantitation limit, } QL = 10\ \sigma / S$$

Where

σ = the standard deviation of the response

S = the slope of calibration curve

f. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. This relationship is evaluated by statistical methods. For the establishment of linearity, a minimum of five concentrations is recommended. The parameters to be calculated are correlation coefficient, y-intercept, slope and residual sum of squares.

g. Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

h. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

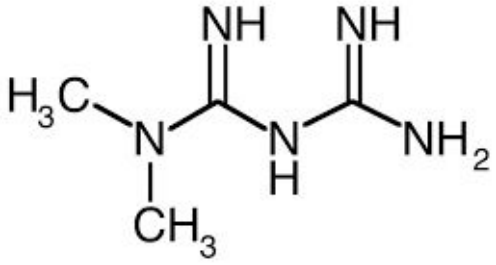
2. LITERATURE REVIEW[9-12]

- Madhukar A. et al. reported a validated HPLC method for analysis of metformin hydrochloride.
- Prasad PBN. et al. proposed a RP – HPLC method for simultaneous determination of metformin and saxagliptin in formulation.
- Sohan SC. et al. reported validated RP - HPLC and derivative spectroscopy methods for determination of teneligliptin.
- Ganesh K. et al. reported RP – HPLC and UPLC tandem mass spectroscopy methods for stability studies and identification of degradation products of teneligliptin.

3. DRUG PROFILE[13-15]

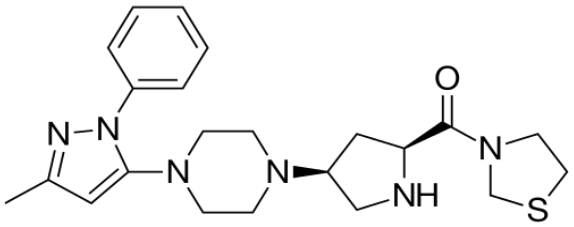
Drug name : Metformin

Drug structure :



IUPAC name	:	N, N – dimethylimidodicarbonimidic diamide
Molecular formula	:	$C_4 H_{11} N_5$
Molecular weight	:	165.62
Description	:	White, crystalline, hygroscopic
Solubility	:	Freely soluble in water; Slightly soluble in ethanol (95%)
Category	:	Type II diabetes mellitus
Strength	:	500 mg

Drug name	:	Teneligliptin
Drug structure	:	



IUPAC name	:	{(2S, 4S)- 4- [4- (3- methyl -1- phenyl - 1H – pyrazol - 5 yl) - 1- piperazinyl] - 2 – pyrolindinyl} (1,3- thiazolindin - 3- yl) methanone
Molecular formula	:	C_{22} H_{30} N_6 OS
Molecular weight	:	426.58
Description	:	White powder
Solubility	:	Soluble in methanol and water
Category	:	Type II diabetes mellitus
Strength	:	20 mg

4. AIM AND PLAN OF WORK

Diabetes is a chronic, metabolic disease characterized by elevated levels of blood glucose (or blood sugar), which leads over time to serious damage to heart, blood vessels, eyes, kidneys and nerves. The most common is type 2 diabetes, usually in adults, which occurs when the body becomes resistant to insulin or doesn't make enough insulin. According to WHO, around 422 million adults have diabetes and 1.5 million deaths are directly attributed to diabetes each year.[16]

Antidiabetic drugs can be used alone or in combination for the management of diabetes. Metformin is an oral anti-hyperglycaemic drug belonging to the class of biguanides which is used in the management of type 2 diabetes.[17] Teneligliptin is a third generation dipeptidyl peptidase-4 inhibitor approved for treatment of type 2 diabetes. Combination of these drugs has been recently launched and it has been reported that teneligliptin co-administered with metformin produced significant reductions in HbA1c in patients with type 2 diabetes mellitus without increasing the risk of hypoglycemia.[18] No HPTLC and HPLC methods have been reported for the simultaneous analysis of these drugs in combined dosage form. Hence, an attempt is made to **develop HPTLC and HPLC methods for the simultaneous determination of metformin and teneligliptin in tablet dosage form.**

5. MATERIALS AND INSTRUMENTS

Active pharmaceutical ingredient (API)

Metformin and Teneligliptin were procured from Tristar Formulations Pvt. Ltd., Pondicherry, India and Zydus Cadila Healthcare Pvt. Ltd., Gujarat, India respectively.

Formulation

ZITA – MET PLUS 20mg/ 500mg extended release tablets**:** Glenmark Pharmaceuticals.

Chemicals and solvents used

- Water- HPLC grade
- Methanol HPLC grade, AR grade
- Tetrahydrofuran - HPLC grade
- Formic acid- AR grade
- Chloroform- LR grade
- Sodium hydroxide- LR grade
- Hydrochloric acid- AR grade
- Hydrogen peroxide- LR grade
- Ammonia 25% solution
- Ammonium acetate
- Potassium dihydrogen phosphate
- Ortho Phosphoric acid
- Hydrochloric acid (0.5 N)
- Sodium hydroxide (0.5 N)
- 30% hydrogen peroxide

All the above chemicals and solvents were supplied by S.D. Fine Chemicals Pvt. Ltd., India, Sigma - Aldrich Chemicals Pvt. Ltd., Maharashtra, India and Ranbaxy chemicals Pvt. Ltd., New Delhi, India.

Stationary phases used

- Pre-coated silica gel $60F_{254}$ on aluminium sheets were procured from Merck, Germany.
- Hibar, C_{18} (250mm X 4.0mm, 5µm) column was obtained from Merck Pvt. Ltd., Mumbai.

Instruments used

- Shimadzu digital electronics balance
- Elico Pvt. Limited, India, pH meter
- Jasco V-600 UV/ Vis- spectrophotometer
- Camag HPTLC system (with TLC Scanner-3, Win CATS software and Linomat V as application device)
- Shimadzu HPLC Prominence *i* LC – 2030 liquid chromatograph system with UV – VISIBLE detector and auto sampler injector. Chromatograms were recorded and integrated on PC installed with Lab solutions chromatographic software.
- Shimadzu liquid chromatograph equipped with LC – 10 AT VP pump, SPD-M10A VP diode array detector and rheodyne 7725 *i* injected with a 20 µl loop. Chromatograms were recorded and integrated on PC installed with LC solutions chromatographic software.

6. EXPERIMENTAL SECTION

I. DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF METFORMIN AND TENELIGLIPTIN IN TABLET DOSAGE FORM

1. Selection of plate

Pre-coated silica gel $60F_{254}$ on aluminium sheet was selected for the study.

2. Selection of solvent

Ideal properties of a solvent employed for HPTLC are

a) Drug should be soluble in the solvent used

b) Drug should show stability in the solvent used

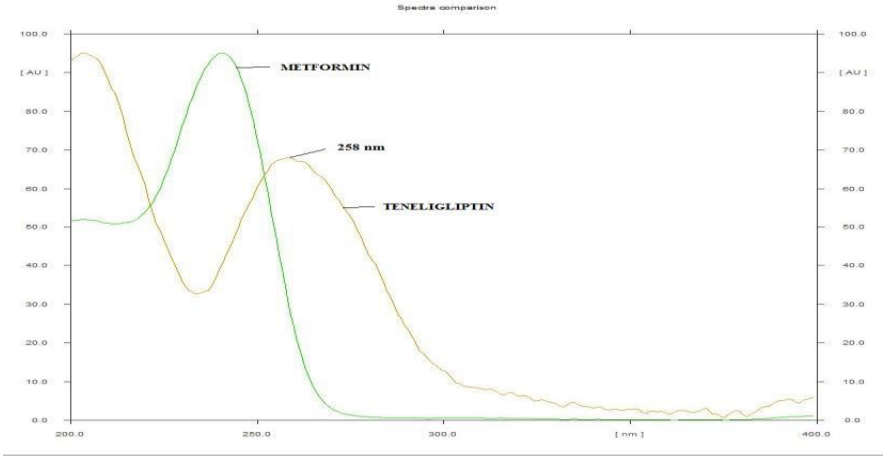
c) Solvent should be volatile

Accordingly, methanol was selected as the solvent.

3. Selection of wavelength

UV spectra of metformin and teneligliptin were recorded on pre-coated TLC plate. The λmax of metformin and teneligliptin were found to be 237 nm and 258 nm respectively. From the overlain UV spectra of the drugs, a wavelength of 258 nm was selected for the study, **fig. 1.**

Fig. 1: Overlain UV spectra of metformin and teneligliptin on precoated TLC plate



4. Developing optimum mobile phase system

A solvent system should be selected in such a way that it would give compact spots and good separation from solvent front and application position. Initially, different solvent systems were tried and observations were as given in **table 1.**

Table: 1 Selection of mobile phase system

SOLVENT SYSTEMS USED	OBSERVATIONS
Chloroform: Methanol: Ammonia [1:9:0.1, v/v/v]	Very low R_f value for metformin (0.06)
Chloroform: Methanol: Water [1:9:0.5, v/v/v]	Teneligliptin moved with solvent front
Ethyl acetate: Methanol: Water: Formic acid [5:3:0.7:1, v/v/v]	Metformin moved with solvent front
Methanol: Tetrahydrofuran: Ammonia [7:3:0.1, v/v/v]	**Compact spots with good separation**

Among these systems, methanol: tetrahydrofuran: ammonia was selected because this system gave good separation with compact spots.

5. Optimization of mobile phase ratio

Different ratios of methanol: tetrahydrofuran: ammonia like 6.5: 2.5: 0.1, 6.5: 2.5: 0.2, 6.5: 2.5: 0.5 and 7: 3: 0.1, v/v/v etc. were tried, from which the ratio of (**7: 3: 0.1, v/v/v)** was selected because it gave compact spots with good separation from solvent front and application positions.

6. Fixed experimental conditions

Sorbent layer	:	Silica gel $60F_{254}$
Mobile phase	:	Methanol: Tetrahydrofuran: Ammonia [7:3:0.1, v/v/v]
Saturation time	:	15 minutes
Total run distance	:	8 cm
Slit dimension	:	5 x 0.45 mm
Plate thickness	:	250 µm
Light source	:	Deuterium lamp
Detection wavelength	:	258 nm
R_f values	:	Metformin 0.29 ± 0.02
		Teneligliptin 0.71 ± 0.02

VALIDATION OF THE METHOD

The validation of the developed method was carried out for various parameters like limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, precision and specificity.

1. Limit of detection [LOD] & Limit of quantification [LOQ]

The LOD and LOQ values were determined by injecting lower concentrations of the drugs. The LOD values for metformin and teneligliptin were found to be 0.01 and 0.1µg/band respectively and their LOQ values were found to be 0.07 and 0.4µg/band respectively, **fig. 2-5.**

Fig. 2: Limit of detection – Metformin

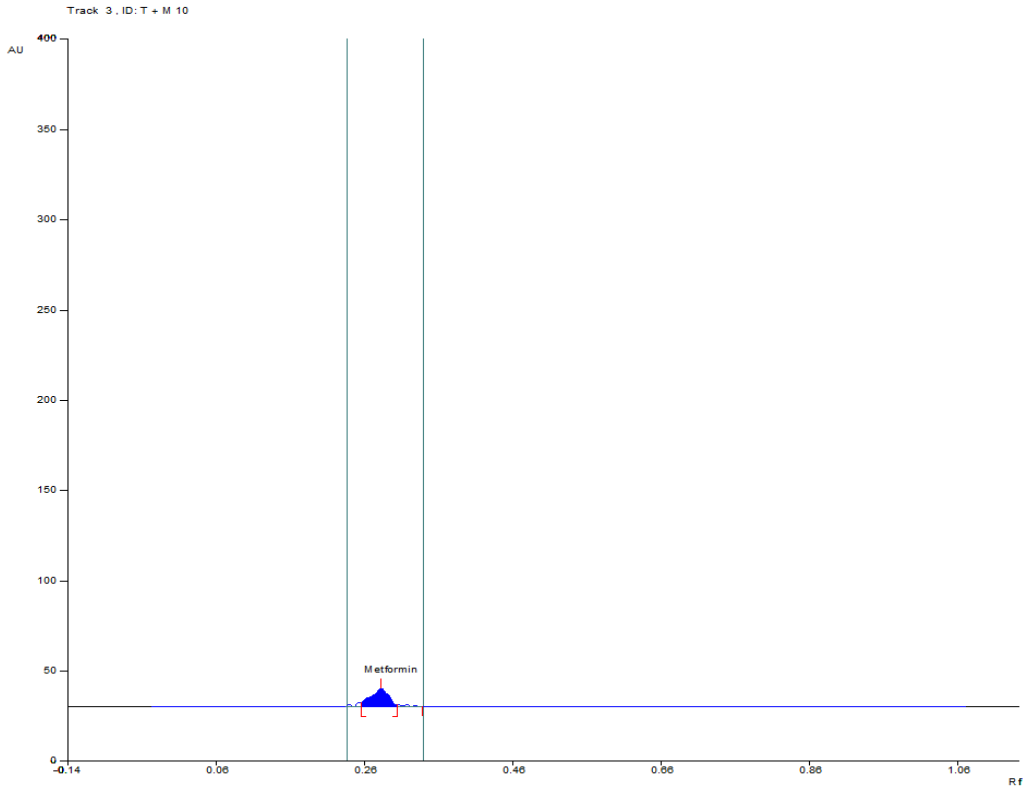


Fig. 3: Limit of detection – Teneligliptin

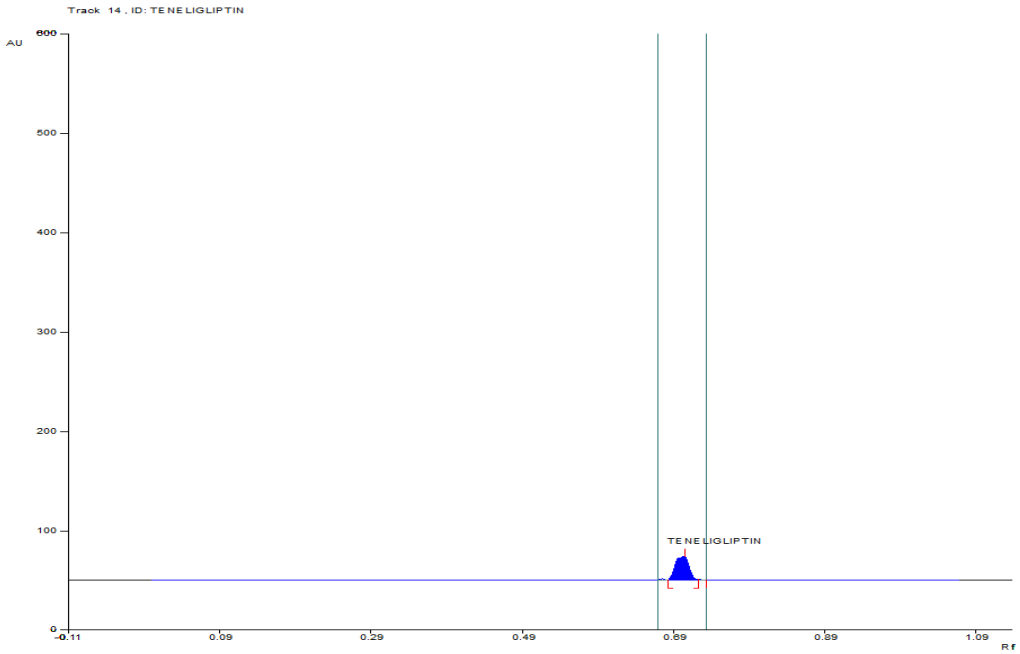


Fig. 4: Limit of quantification – Metformin

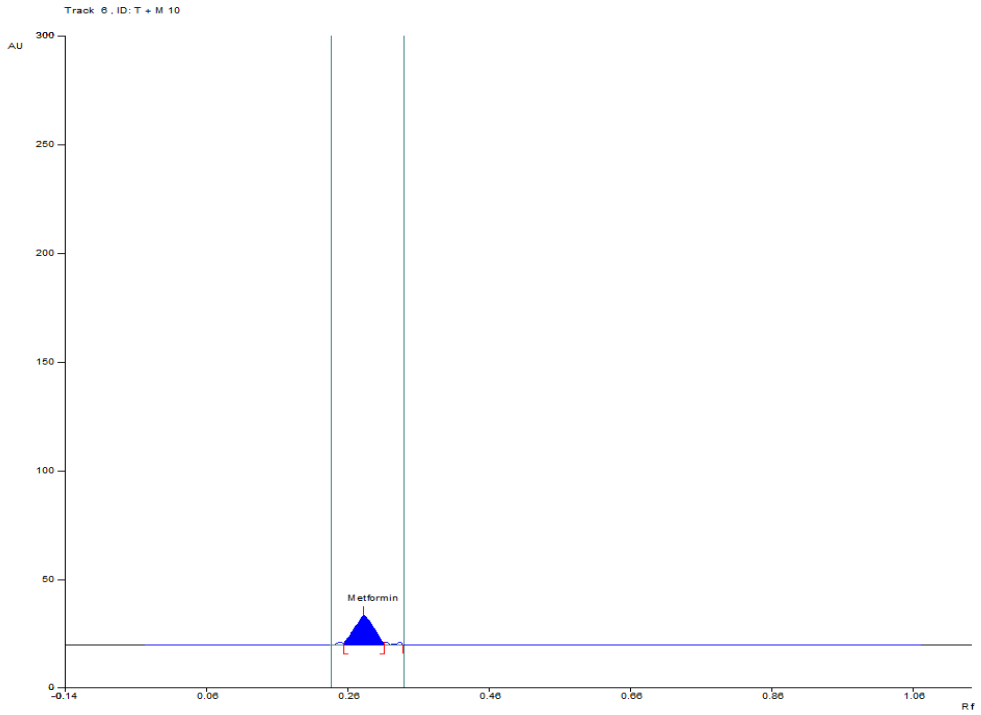
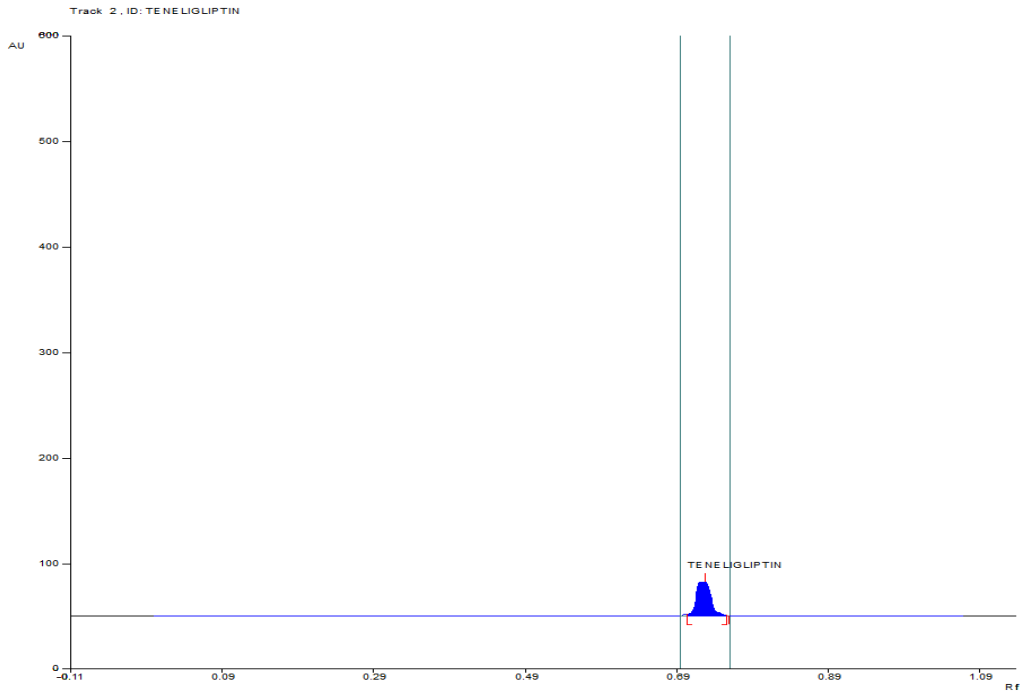


Fig. 5: Limit of quantification – Teneligliptin



2. Linearity

a) Metformin

Metformin was found to be linear in the concentration range of 6.25-17.5 µg/band. Calibration curve was plotted using concentration (x) versus peak area (y). The slope, intercept and correlation coefficient values were found to be 1132.521, 6055.128 and 0.9991 respectively, **fig. 6; table 2.**

The regression equation is as follows

Peak area = 6055.128 + 1132.521 x concentration

Fig. 6: Calibration graph of metformin

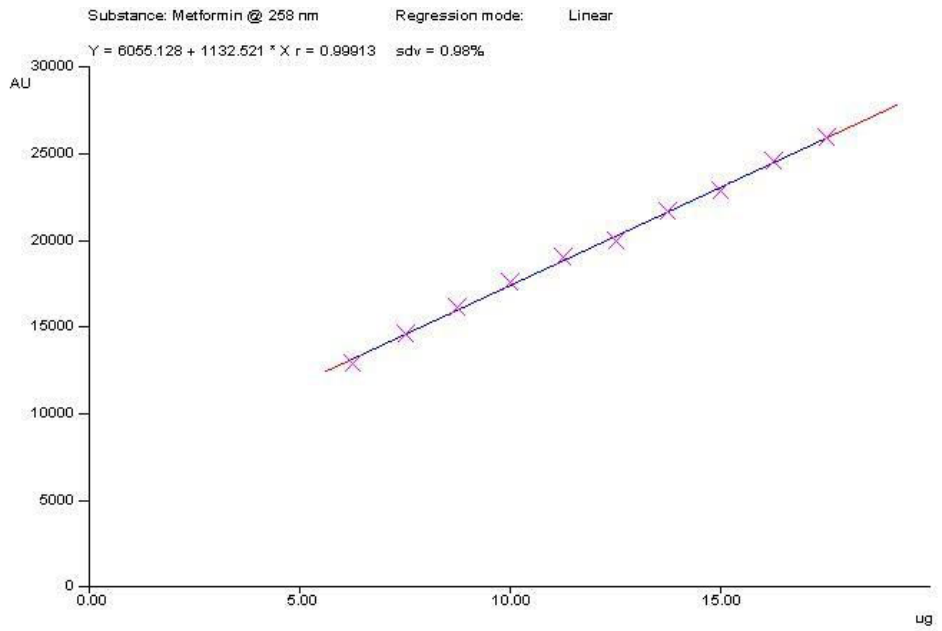


Table 2: Calibration data (6.25 – 17.50 µg/band)

Concentration (µg/band)	Peak Area
6.25	13296.8
7.5	14965.5
8.75	16384.9
10.00	17390.0
11.25	19311.4
12.50	20268.3
13.75	21735.8
15.00	23118.7
16.25	24468.4
17.5	25306.2

b) Teneligliptin

Teneligliptin was found to be linear in the concentration range of 0.25-0.7 µg/band. Calibration curve was plotted using concentration (x) versus peak area (y). The slope, intercept and correlation coefficient values were found to be 2.725, -125.859 and 0.9981 respectively, **fig. 7; table 3.**

The regression equation is as follows

Peak area = (-125.859) + 2.725x concentration

Fig. 7: Calibration graph of teneligliptin

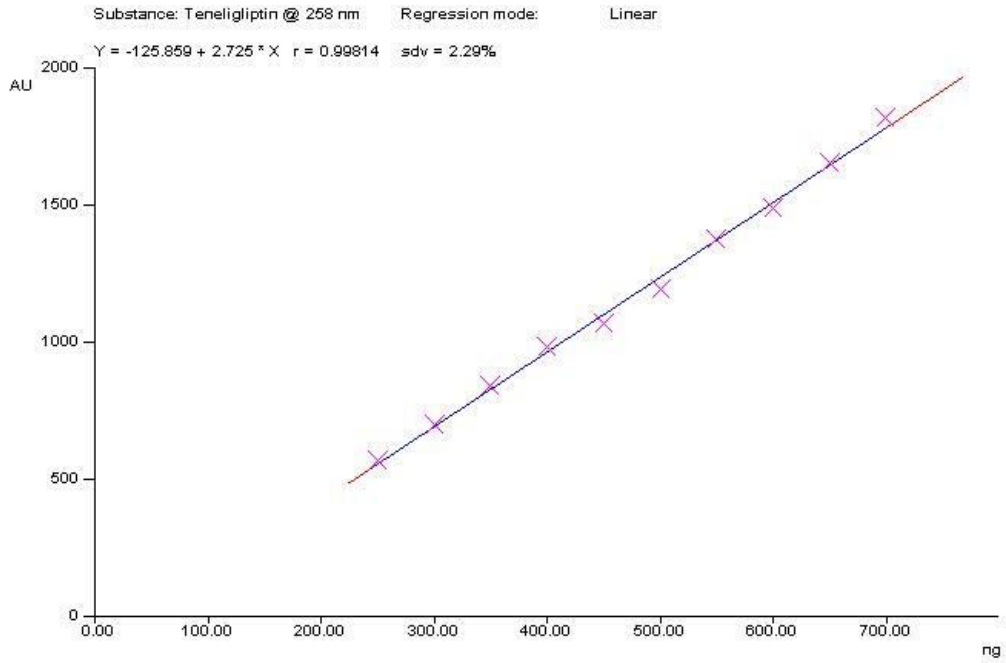


Table 3: Calibration data (0.25 – 7 µg/band)

Concentration (µg/band)	Peak Area
0.25	624.7
0.30	745.4
0.35	881.3
0.40	993.5
0.45	1188.9
0.50	1278.4
0.55	1449.0
0.60	1606.3
0.65	1722.1
0.70	1891.7

3. Accuracy

Recovery studies were done for determining accuracy parameter. It was done by mixing known quantity of standard drug with the analysed sample formulation and the contents were reanalysed by the proposed method. Recovery studies were carried out at 80, 100 and 120% levels. The percentage recovery and its %RSD were calculated, **table 4 and 5.**

Table 4: Recovery studies for metformin

Level	%Recovery	%RSD*
80%	99.62	0.6
100%	101.25	0.37
120%	100.92	0.54

***RSD of 6 observations**

Table 5: Recovery studies for teneligliptin

Level	%Recovery	%RSD*
80%	99.99	0.19
100%	100.98	0.64
120%	101.06	0.64

***RSD of 6 observations**

4. Precision

Precision of method was demonstrated by:

i.) Intra-day precision

ii.) Inter-day precision

iii.) Repeatability

a) Repeatability of sample application

b) Repeatability of sample measurement

i) Intraday precision

Intraday precision was studied by carrying out the analysis of the standard drugs at two different concentrations in the linearity range of the drugs for three times on the same day and %RSD was calculated, **table 6.**

Table 6: Intraday precision

Concentration	Peak Area		% RSD	
	Metformin	Teneligliptin	Metformin	Teneligliptin
10.00(µg/band) Metformin 0.4(µg/band) Teneligliptin	17505.5	969.3	0.11	0.43
	17491.6	950.5		
	17467.2	944.8		
12.50(µg/band) Metformin 0.5(µg/band) Teneligliptin	19864.2	1171.0	1.3	1.7
	19692.6	1130.9		
	19796.0	1157.5		

ii) Inter-day precision

Inter-day precision was studied by carrying out the analysis of the standard drugs at two different concentrations in the linearity range of the drugs for three days over a period of one week and % RSD was calculated, **table 7.**

Table 7: Inter-day precision

Concentration	Peak Area		% RSD	
	Metformin	Teneligliptin	Metformin	Teneligliptin
10.00(µg/band) Metformin 0.4(µg/band) Teneligliptin	17593.2	983.3	0.54	1.26
	17467.2	958.8		
	17406.0	973.1		
12.50(µg/band) Metformin 0.5(µg/band) Teneligliptin	19973.1	1195.0	0.44	1.43
	19796.0	1157.5		
	19872.8	1198.5		

iii) Repeatability

a) Repeatability of sample application

Repeatability of sample application was evaluated by spotting drug solution six times on pre-coated TLC plate. Plate was then developed, scanned and %RSD was calculated, **table 8.**

Table 8: Repeatability of sample application

Concentration	Peak Area		% RSD	
	Metformin	Teneligliptin	Metformin	Teneligliptin
10.00(µg/band) Metformin 0.4(µg/band) Teneligliptin	17988.3	956.2	0.13	0.7
	17982.8	974.6		
	17927.0	963.6		
	17960.1	959.5		
	17941.8	956.8		
	17960.3	960.3		

b) Repeatability of sample measurement

Repeatability of measurement of peak area was evaluated by spotting the standard drug solutions on pre-coated TLC plate. After development of the plate the separated spots were scanned six times without changing the position of the plate and % RSD was calculated, **table 9.**

Table 9: Repeatability of sample measurement

Concentration	Peak Area		% RSD	
	Metformin	Teneligliptin	Metformin	Teneligliptin
10.00(µg/band) Metformin 0.4(µg/band) Teneligliptin	17799.0	986.9	0.22	1.37
	17709.8	988.1		
	17770.9	994.3		
	17755.3	969.9		
	17696.7	959.2		
	17771.3	988.1		

5. Specificity

The peak purity of metformin and teneligliptin was assessed by comparing their respective spectra at peak start, peak apex and peak end positions of the spot. The good correlation among spectra acquired at start (s), apex (m) and end (e) of the peaks metformin[{correlation r(s,m)=0.99943}, r(m,e)=0.99908}] and teneligliptin [{correlation r(s,m) =0.99966}, r(m,e) =0.99521}] indicates good peak purities of drugs, **fig. 8 and 9.** It can be concluded that no impurities or degradation products migrated with the peaks obtained from standard solutions of the drugs.

Fig. 8: Peak purity graph showing correlation between peak maxima and slope for metformin

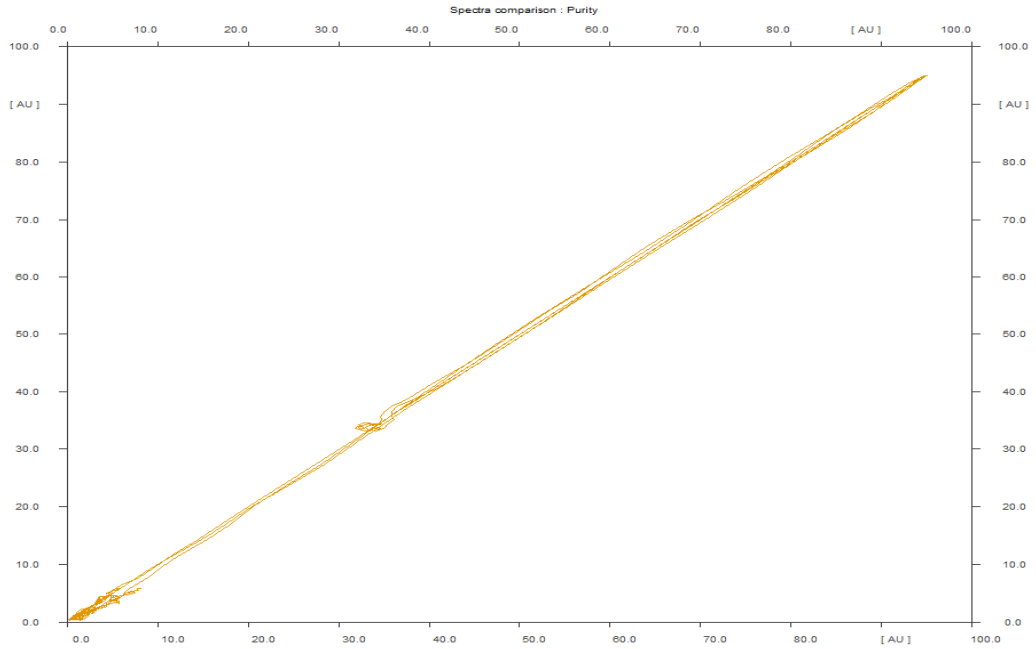
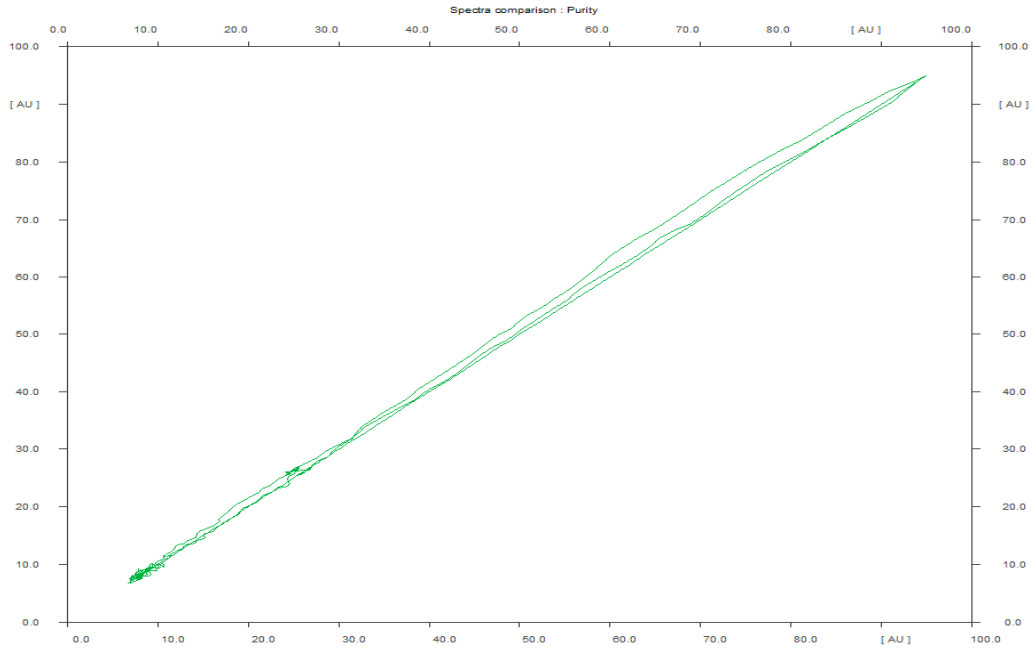


Fig. 9: Peak purity graph showing correlation between peak maxima and slope for teneligliptin



ANALYSIS OF FORMULATION

1. Preparation of stock solution

A stock solution of metformin (2500 µg/ml) and teneligliptin (100 µg/ml) was prepared in methanol.

2. Preparation of sample solutions

Ten tablets each containing 500 mg of metformin and 20 mg of teneligliptin were weighed and the average weight was determined. Amount of powder equivalent to 125 mg of metformin and 5 mg of teneligliptin was transferred to a 50 ml volumetric flask, and 25 ml methanol was added. The contents of the flask were shaken for 10 minutes, followed by dilution to volume with methanol to provide a solution containing 2500 µg/ml of metformin and 100 µg/ml of teneligliptin. This solution was filtered through a 0.45 mm membrane filter before injection.

3. Recording the chromatograms

With the fixed chromatographic conditions, 2.5 – 7 µl (i.e. 6.25 to 17.5 µg/band for metformin and 0.25 to 0.7 µg/band for teneligliptin) from standard stock solution and suitable volumes from sample solution were applied on the precoated TLC plate. The plates were analysed and chromatograms were recorded and results are given in **table 10.** The standard chromatograms, overlain UV spectra of drugs in formulation and 3D image of standard chromatograms are given in **fig. 10 – 21.**

Table 10: Analysis of formulation

Drug	Amount of drug (mg/tablet)		% Label claim	%RSD*
	Labelled	Found		
Zita met plus (Teneligliptin 20 mg & Metformin 500mg)	20 mg	19.84 mg	99.2	0.98
	500 mg	491.4 mg	99.3	1.12

***RSD of 6 observations**

CHROMATOGRAMS OF STANDARDS

Fig. 10: Chromatogram of standard 1 (MET 6.25 µg/band; TENE 0.25 µg/band)

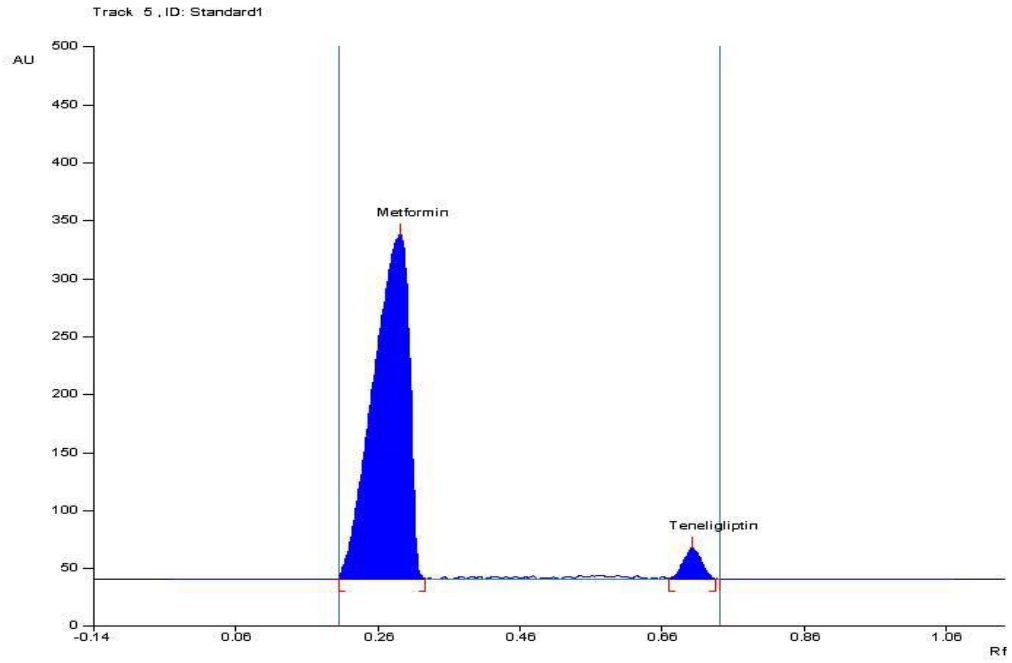


Fig. 11: Chromatogram of standard 2 (MET 7.5 µg/band; TENE 0.3 µg/band)

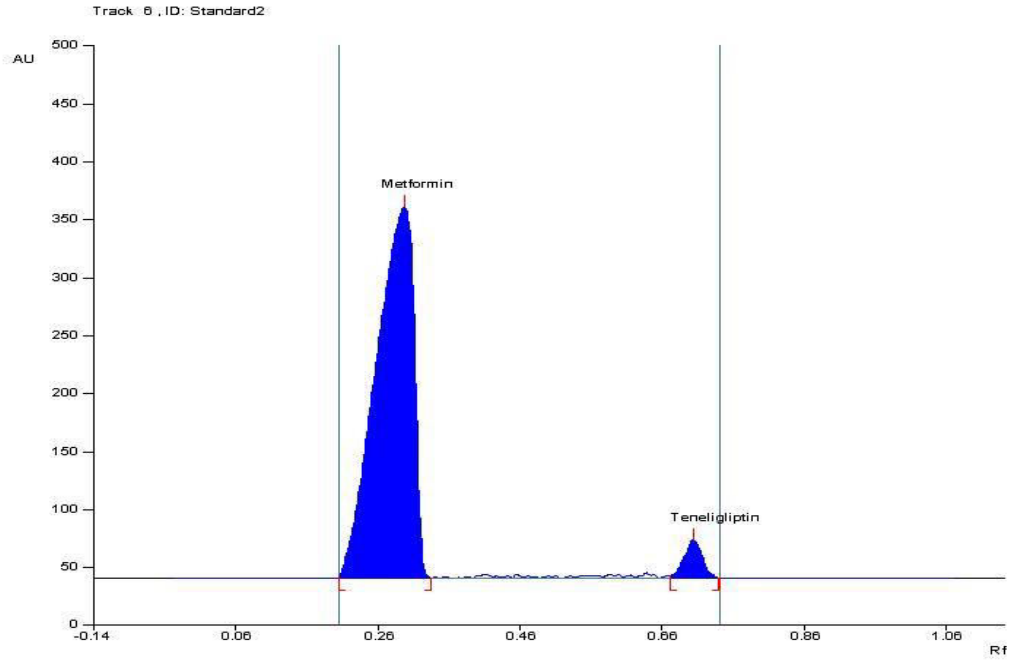


Fig. 12: Chromatogram of standard 3 (MET 8.75 µg/band; TENE 0.35 µg/band)

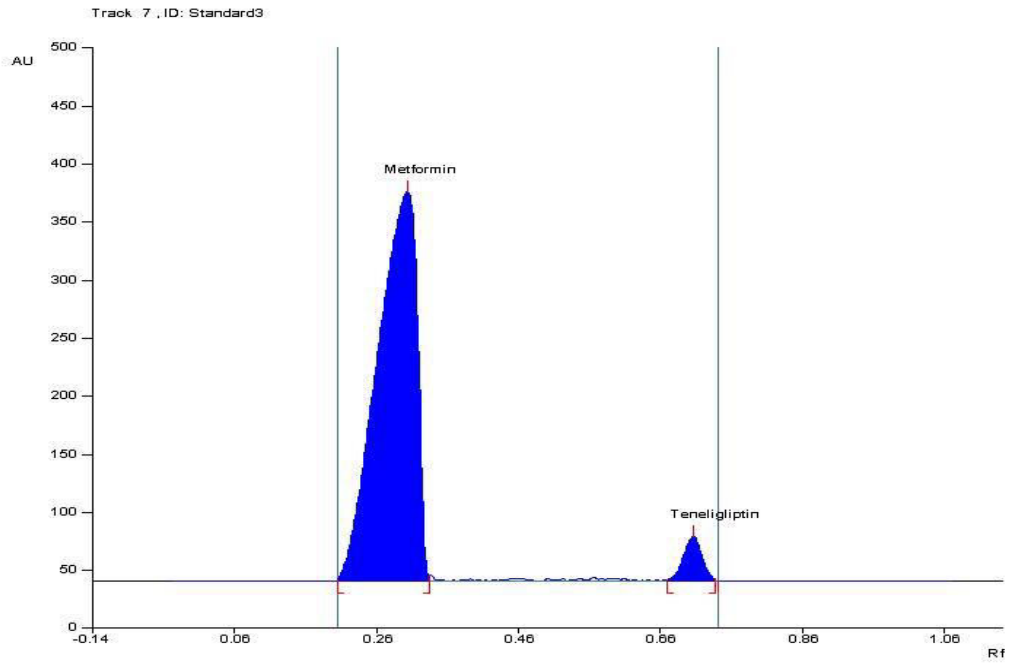


Fig. 13: Chromatogram of standard 4 (MET 10.00 µg/band; TENE 0.4 µg/band)

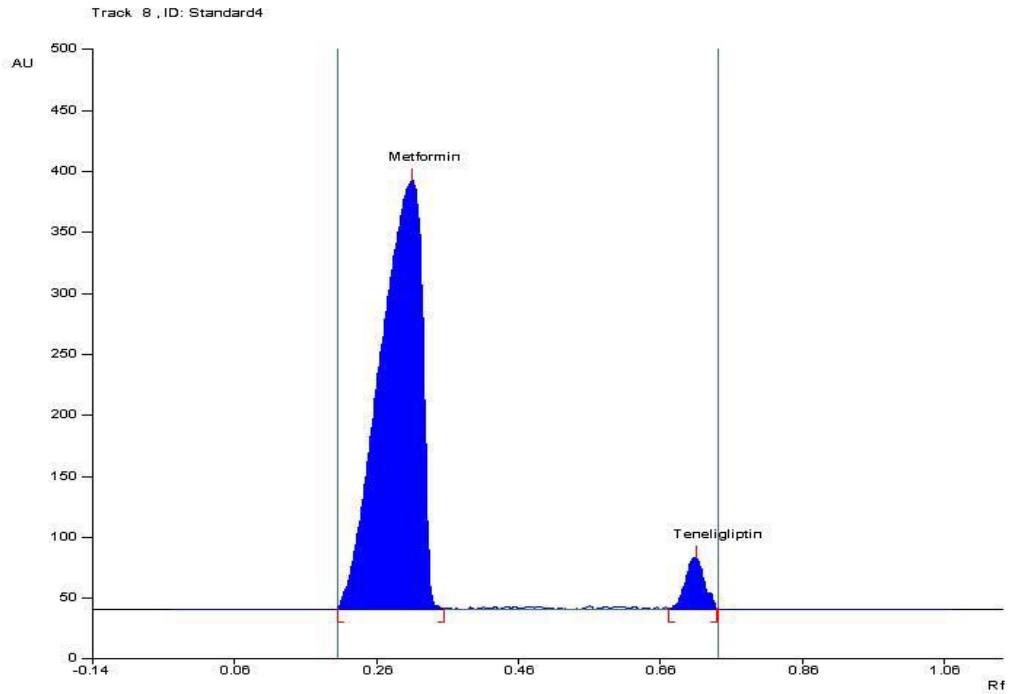


Fig. 14: Chromatogram of standard 5 (MET 11.25µg/band; TENE 0.45µg/band)

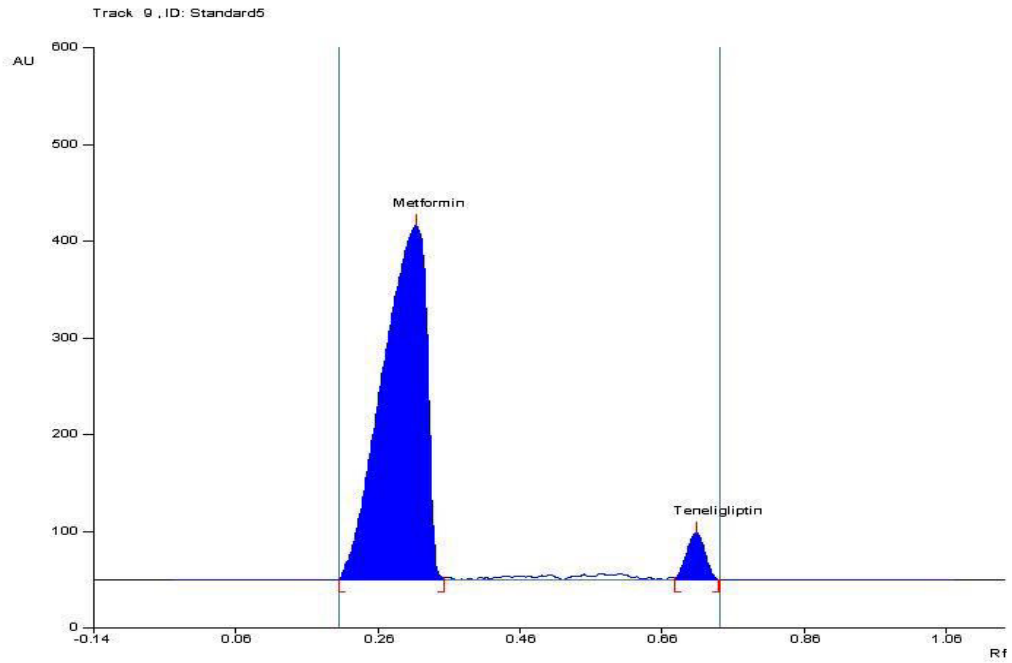


Fig. 15: Chromatogram of standard 6 (MET 12.5 µg/band; TENE 0.5 µg/band)

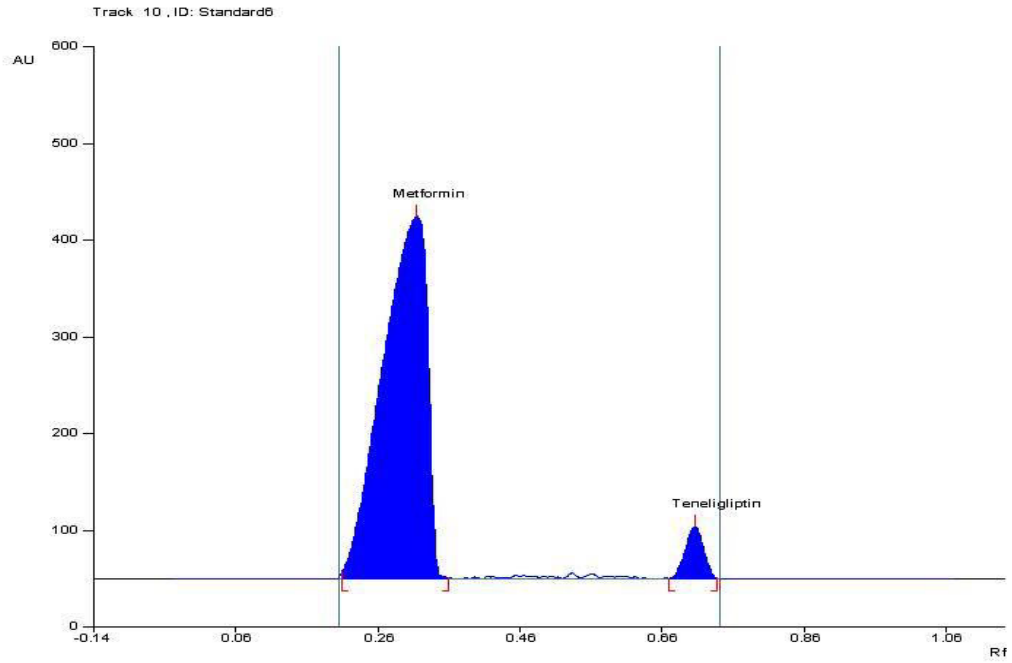


Fig. 16: Chromatogram of standard 7 (MET 13.75µg/band; TENE0.55µg/band)

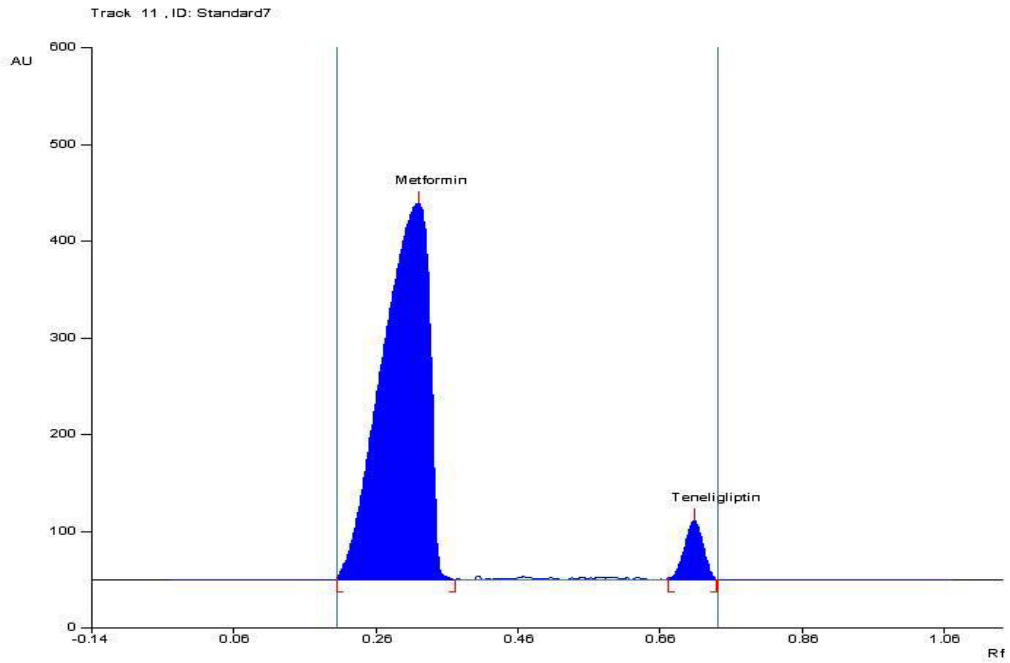


Fig. 17: Chromatogram of standard 8 (MET 15.00µg/band; TENE 0.6 µg/band)

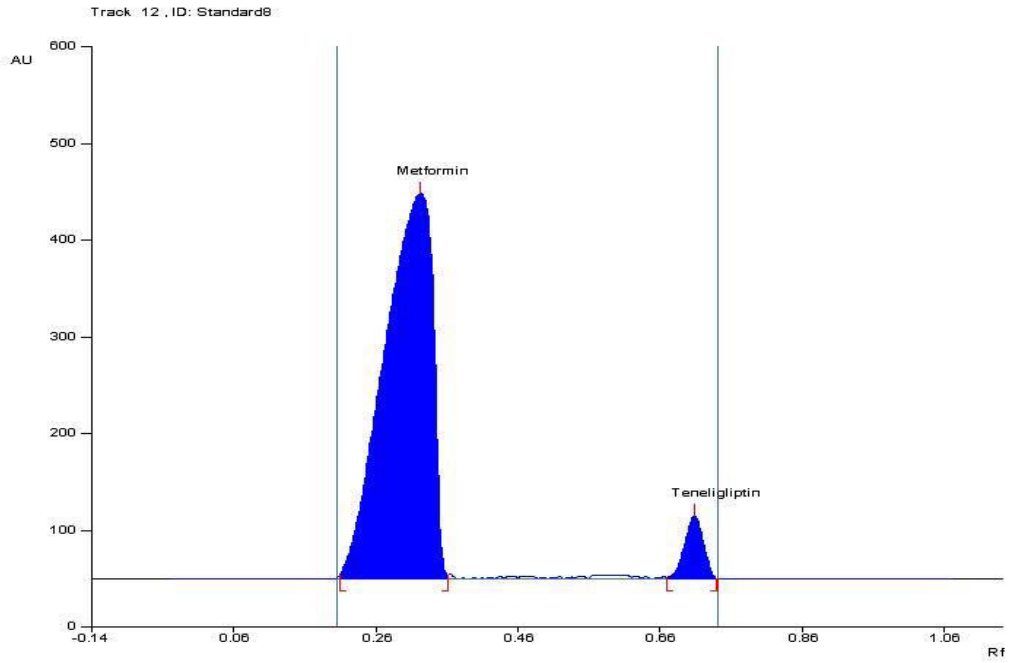


Fig. 18: Chromatogram of standard 9 (MET 16.25µg/band; TENE0.65µg/band)

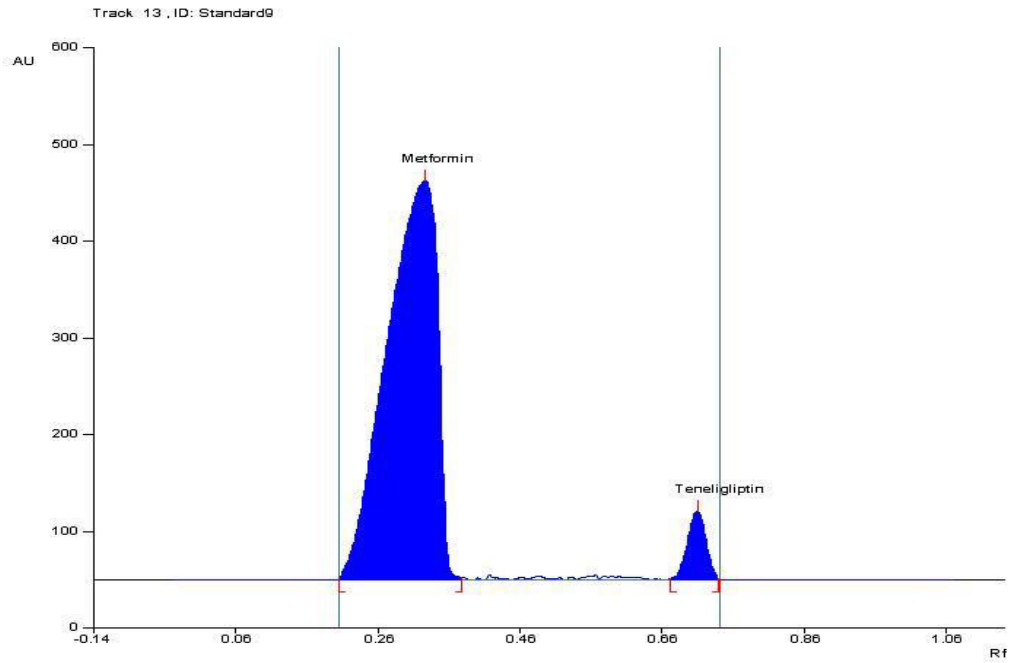


Fig. 19: Chromatogram of standard 10 (MET 17.5µg/band; TENE 0.7µg/band)

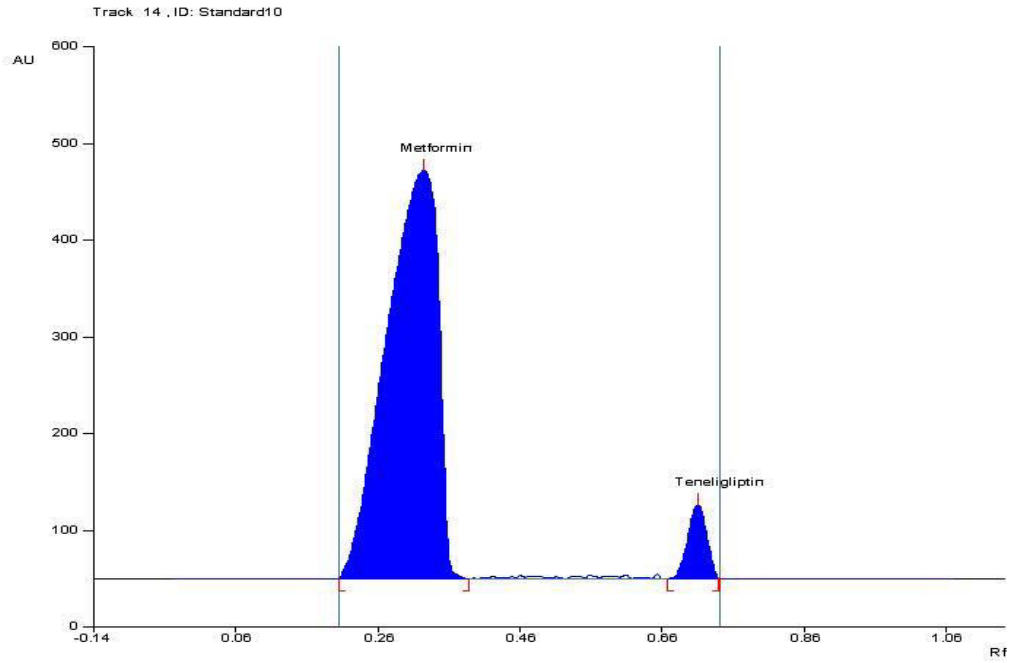


Fig. 20: Overlain UV spectra of drugs in formulation on precoated TLC plate

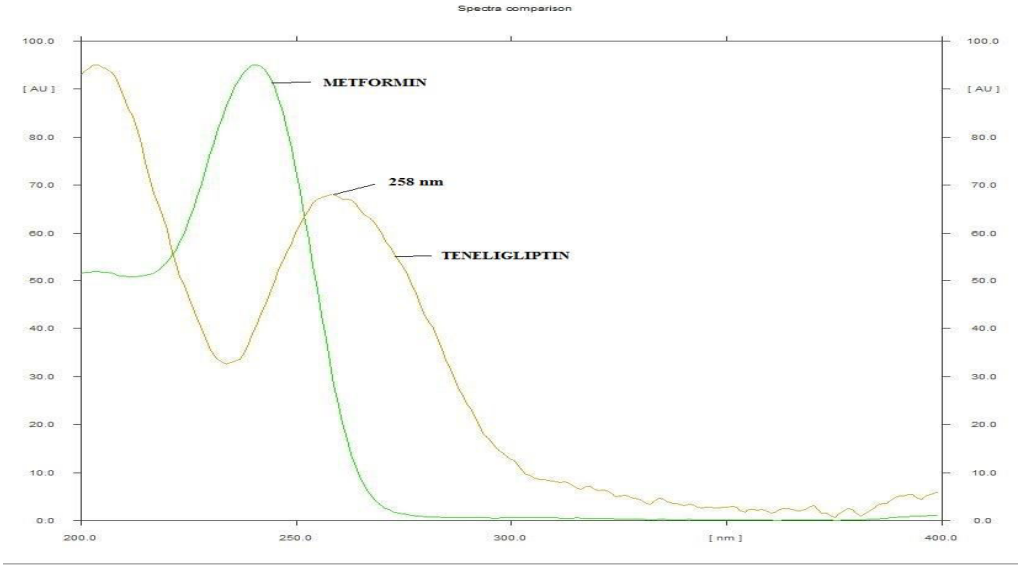
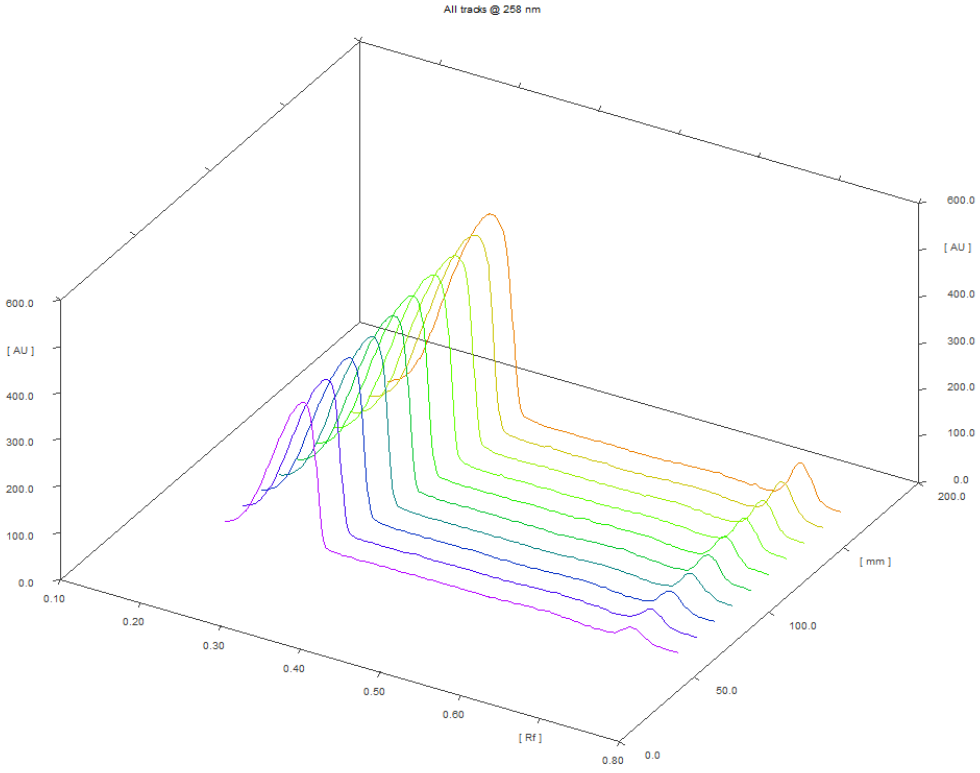


Fig. 21: 3D Image of standard chromatograms



II. DEVELOPMENT OF VALIDATED RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF METFORMIN AND TENELIGLIPTIN IN TABLET DOSAGE FORM

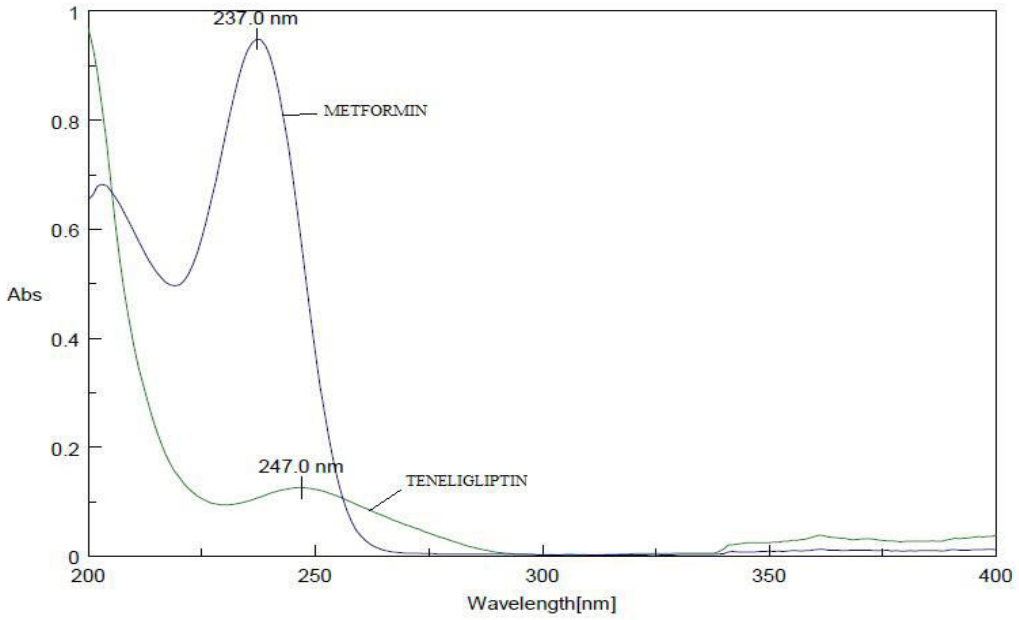
1. Selection of stationary phase

Since metformin and teneligliptin are polar in nature, RP-HPLC method with C_{18} column was selected for method development.

2. Selection of wavelength

Selectivity of HPLC method that uses UV detector depends on proper selection of wavelength. A wavelength which gives good response for the drugs to be detected is to be selected. From UV spectral studies, 247 nm was selected as detection wavelength for metformin and teneligliptin, **fig. 22**.

Fig. 22: Overlain UV spectra of metformin and teneligliptin



3. Selection of mobile phase

Solvent type, solvent strength, strength of buffer and optimum pH were optimised to get the chromatographic conditions that gave best separation, **fig. 23-30; table 11.**

Table 11: Selection of mobile phase

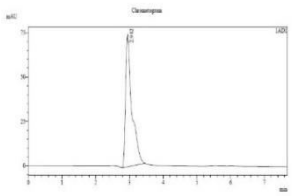
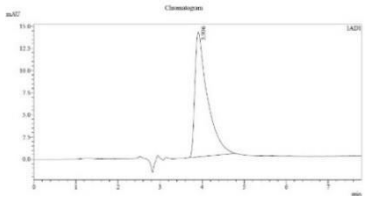
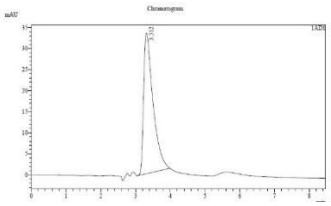
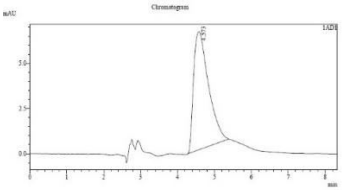
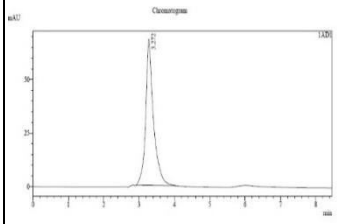
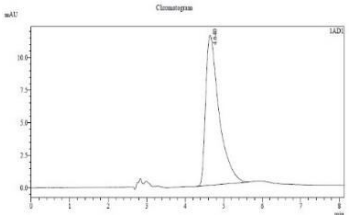
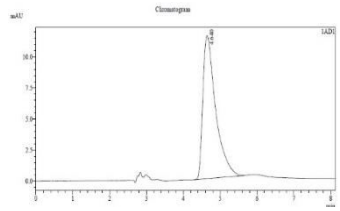
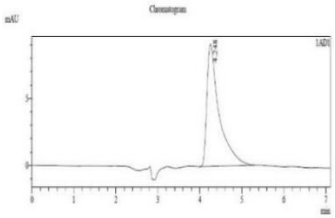
Figure No.	Mobile phase	Chromatograms	
		Metformin	Teneligliptin
23 & 24	Water: Methanol	 **Asymmetric peak**	 **Peak tailing**
25 & 26	10 mM Sodium dihydrogen Ortho phosphate: Methanol	 **Peak tailing**	 **Broad peak**
27 & 28	10 mM Potassium dihydrogen Ortho phosphate: Methanol	 **Peak shape good**	 **Good symmetrical peak**

Figure No.	Mobile phase	Chromatograms	
		Metformin	Teneligliptin
29 & 30	10 mM Ammonium acetate: Methanol	 Peak tailing	 Peak tailing

4. Selection of mobile phase ratio

Mobile phase system containing 10 mM potassium dihydrogen ortho phosphate and methanol was tested in different ratios like 30: 70, 35: 65 and 40: 60 v/v. A ratio of 35: 65% v/v gave good resolution and peak characteristics, **fig. 31-33; table 12.**

Table 12: Selection of mobile phase ratio

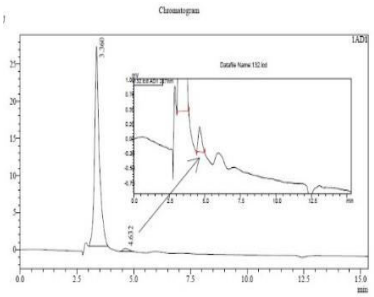
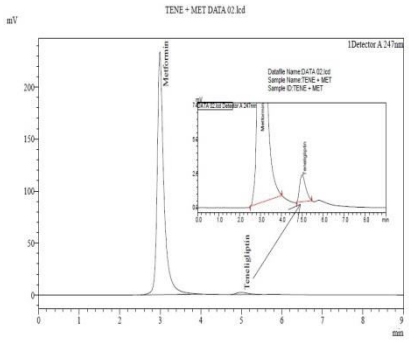
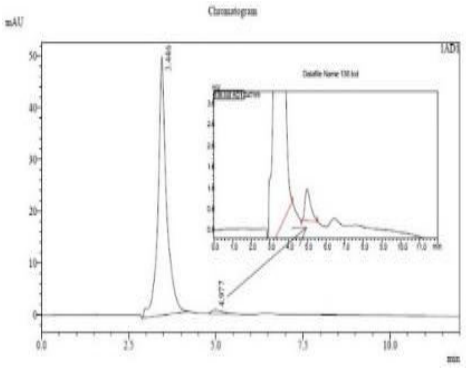
Figure No.	Mobile phase ratio	Chromatogram	Observation
31	30:70		Less resolution between the drug peaks

Figure No.	Mobile phase ratio	Chromatogram	Observation
32	35: 65		Good peak shape and acceptable resolution
33	40: 60		Drug peak shapes not good

5. Selection of pH

Different pH's of 10 mM potassium dihydrogen orthophosphate such as 3, 3.5, 4, 4.5 and 5.4 were tried. Good peak characteristics was observed for pH 4 and hence selected for further studies, **fig. 34–38; table 13.**

Table 13: Selection of pH

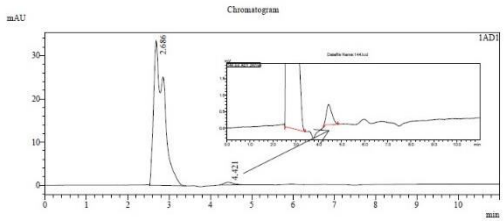
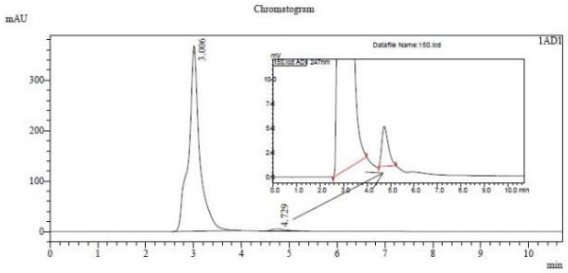
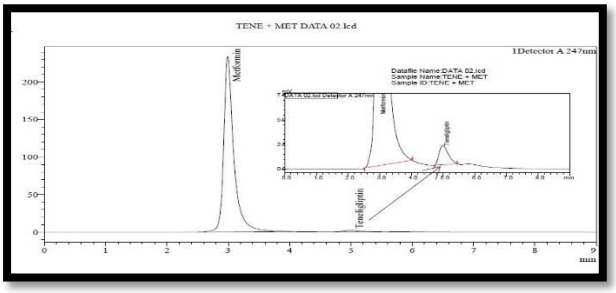
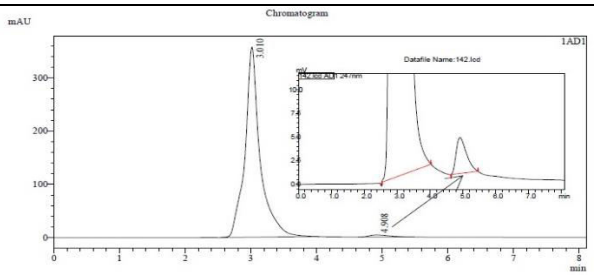
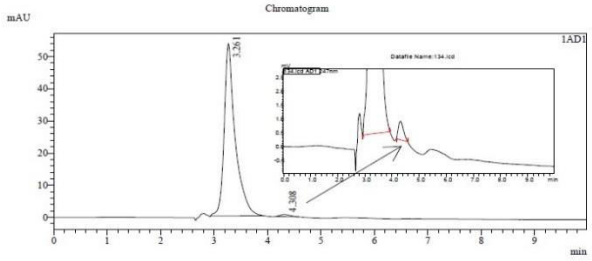
Figure No.	pH	Chromatogram	Observation
34	3.0		Split peak obtained for metformin
35	3.5		Peak asymmetry for metformin
36	**4.0**		**Good peak shapes**
37	4.5		Asymmetric peak shape

Figure No.	pH	Chromatogram	Observation
38	5.4		Less resolution between the drug peaks

6. Fixed chromatographic conditions

Stationary phase : Hibar, C_{18} column (250mm X 4.0mm, 5µm)

Mobile phase : 10 mM potassium dihydrogen orthophosphate (adjusted to pH 4 using ortho phosphoric acid): Methanol

Solvent ratio : 35: 65, v/v

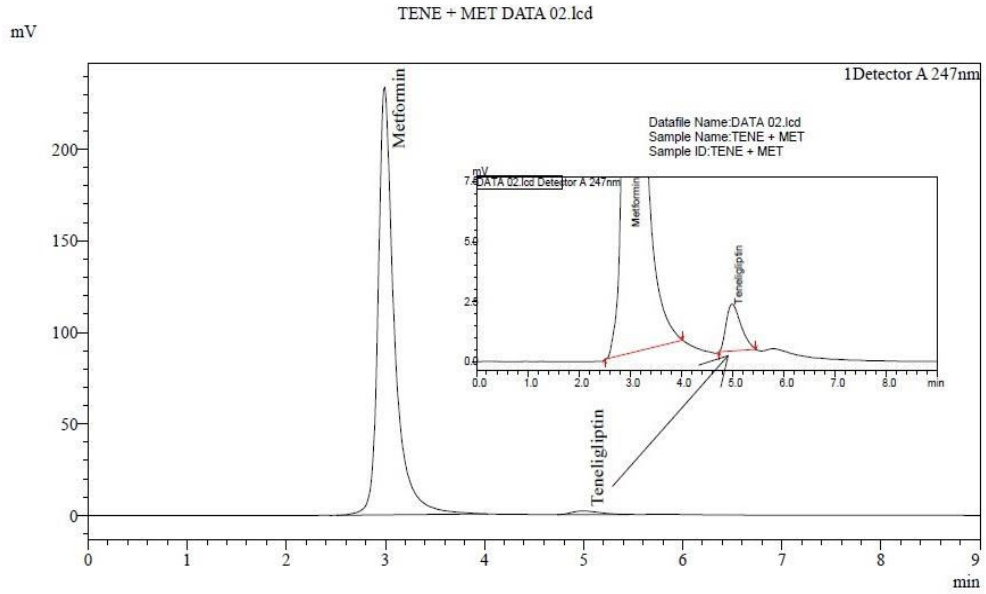
Flow rate : 1.0 ml/min

Detection wavelength : 247 nm

Temperature : Room temperature

A chromatogram showing the separation of metformin and teneligliptin using the fixed chromatographic conditions is shown in **fig. 39.**

Fig. 39: A chromatogram of metformin and teneligliptin using fixed chromatographic conditions



METHOD VALIDATION

The validation of the developed method was carried out for various parameters like linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), stability, robustness and specificity.

1. Limit of detection and limit of quantification (LOD & LOQ)

The LOD and LOQ values were determined by injecting lower concentrations of the drugs. The LOD values for metformin and teneligliptin were found to be 5 and 10 ng/ml respectively and their LOQ values were found to be 10 and 70 ng/ml respectively, **fig. 40-43**

Fig. 40: LOD - Metformin

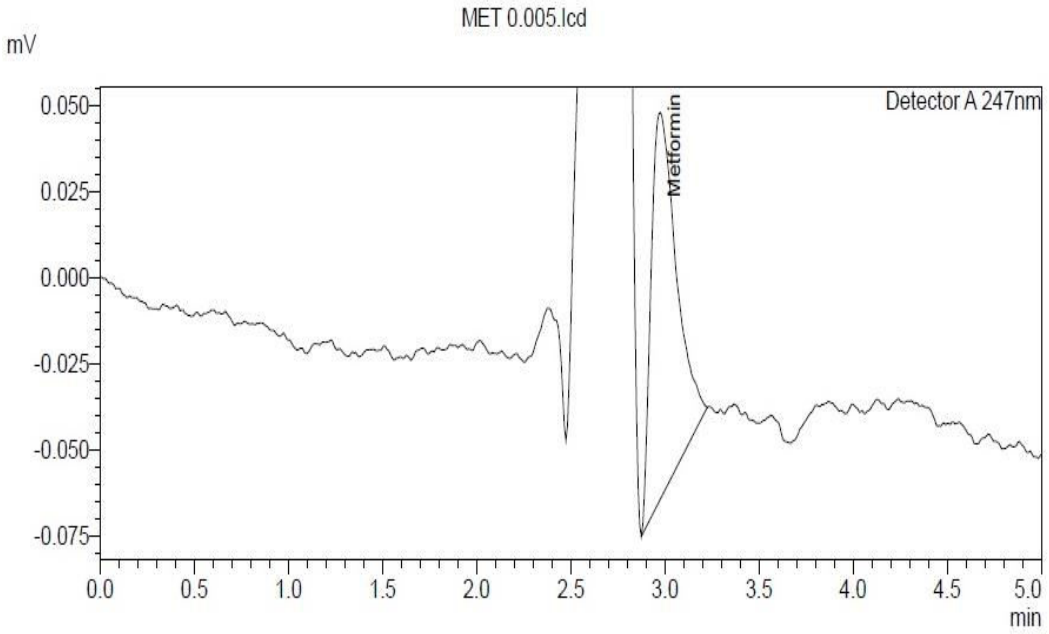


Fig. 41: LOD - Teneligliptin

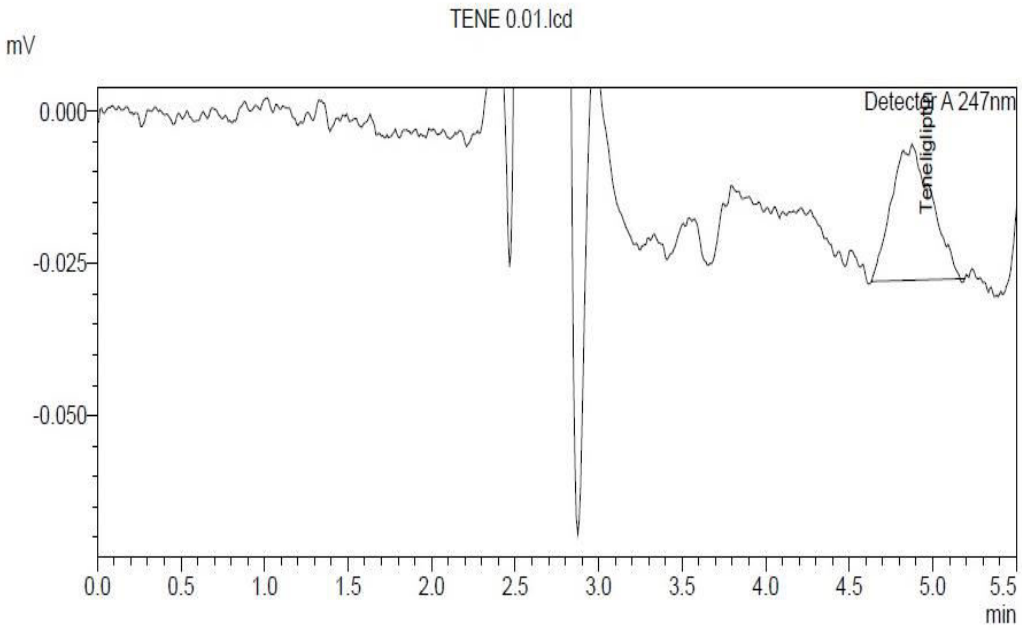


Fig. 42: LOQ – Metformin

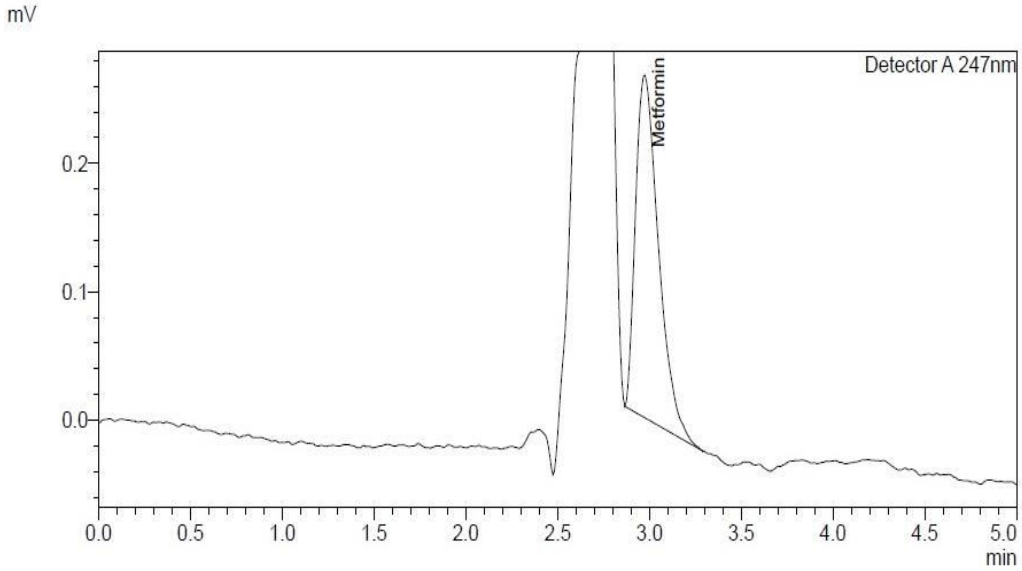
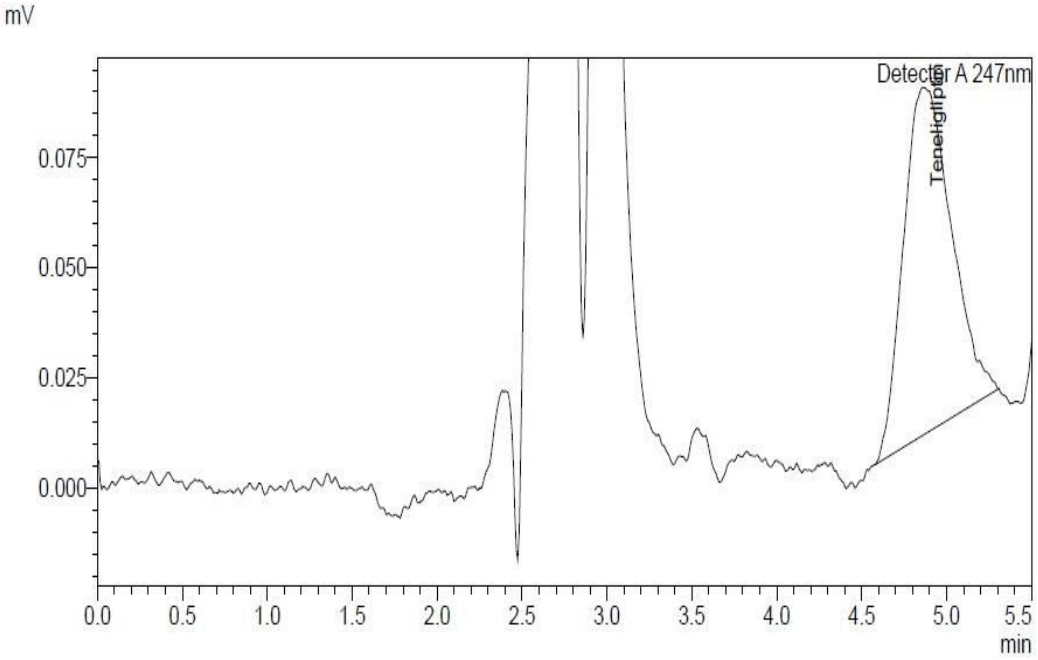


Fig. 43: LOQ – Teneligliptin



2. Linearity

a) Metformin

Metformin was found to be linear in the concentration range of 25 – 250 µg/ml. Calibration curve was plotted using concentration (x) versus peak area (y). The slope, intercept and correlation coefficient values were found to be 46749.2, 281930 and 0.9998 respectively, **fig. 44; table 14.**

The regression equation is as follows

Peak area = 281930 + 46749.2 x concentration

Fig. 44: Calibration graph – Metformin

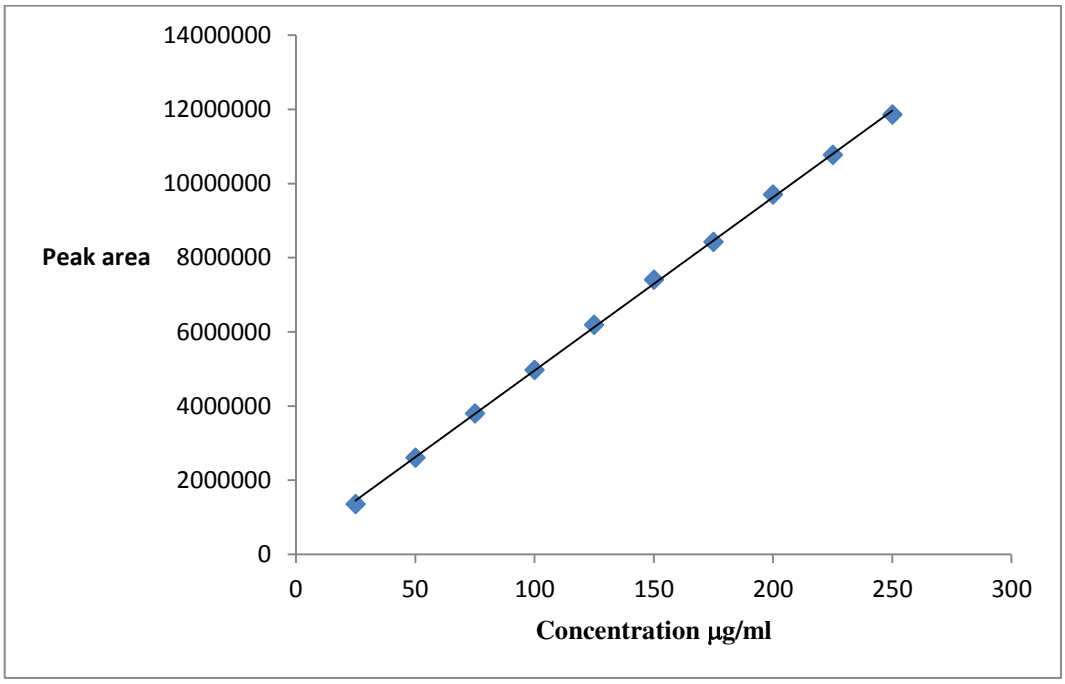


Table 14: Metformin - Calibration data (25 – 250 µg/ml)

Concentration (µg/ml)	Peak Area
25	1354287
50	2611634
75	3796175
100	4972965
125	6188947
150	7408120
175	8428047
200	9700838
225	10771794
250	11866670

b) Teneligliptin

Teneligliptin was found to be linear in the concentration range of 1 – 10 µg/ml. Calibration curve was plotted using concentration (x) versus peak area (y). The slope, intercept and correlation coefficient values were found to be 16392.2, -1954.47 and 0.9991 respectively, **fig. 45; table 15.**

The regression equation is as follows

Peak area = (-1954.47) + 16392.2 x concentration

Fig. 45: Calibration graph - Teneligliptin

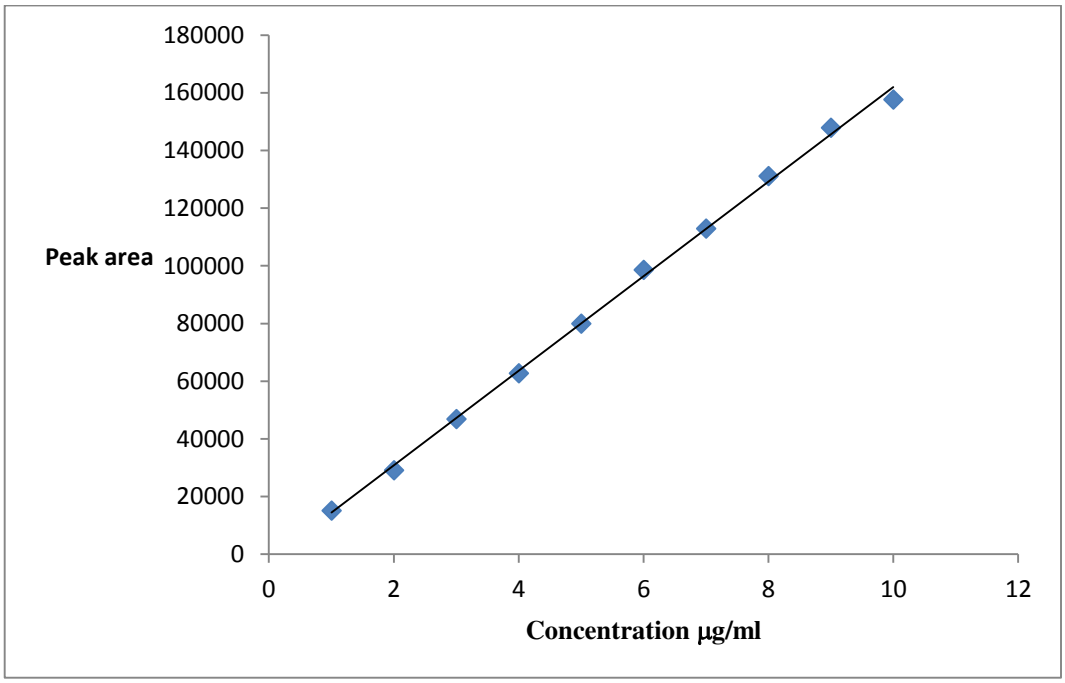


Table 15: Teneligliptin - Calibration data (1 – 10 µg/ml)

Concentration (µg/ml)	Peak Area
1	15169
2	29070
3	46856
4	62792
5	79957
6	98550
7	112943
8	131102
9	147915
10	157651

3. Accuracy

Recovery studies were done for determining accuracy parameter. It was done by spiking known quantity of standard drug with the analysed sample formulation and the contents were reanalysed by the proposed method. Recovery studies were carried out at 80, 100 and 120% levels. The percentage recovery and its %RSD were calculated, **table 16 & 17.**

Table 16: Recovery studies for metformin

Level	%Recovery	%RSD*
80%	99.9	0.71
100%	99.78	0.77
120%	103.81	0.77

***RSD of 6 observations**

Table 17: Recovery studies for teneligliptin

Level	%Recovery	%RSD*
80%	99.43	0.7
100%	100.27	0.57
120%	99.66	0.81

***RSD of 6 observations**

4. Precision

Precision of method was demonstrated by:

i.) Intraday precision

ii.) Inter-day precision

iii.) Repeatability

a. Repeatability of injection

i) Intraday precision

Intra-day precision was studied by carrying out the analysis of the standard drugs at two different concentrations in the linearity range of the drugs for three times on the same day and %RSD was calculated, **table 18.**

Table 18: Intraday precision

Concentration	Peak Area		%RSD	
	Metformin	Teneligliptin	Metformin	Teneligliptin
100 (µg/ml) Metformin 4 (µg/ml) Teneligliptin	5123139	60866	0.88	0.46
	5173112	61212		
	5214763	61427		
125 (µg/ml) metformin 5 (µg/ml) Teneligliptin	6299618	78644	0.98	0.29
	6279424	79109		
	6184767	78945		

ii) Inter-day precision

Inter-day precision was studied by carrying out the analysis of the standard drugs at two different concentrations in the linearity range of the drugs for three days over a period of one week and %RSD was calculated, **table 19.**

Table 19: Inter-day precision

Concentration	Peak Area		% RSD	
	Metformin	Teneligliptin	Metformin	Teneligliptin
100 (µg/ml) Metformin 4 (µg/ml) Teneligliptin	5172965	62792	0.65	0.19
	4967626	62553		
	5027176	62720		
125 (µg/ml) Metformin 5 (µg/ml) Teneligliptin	6188947	79957	0.71	1.15
	6203468	79833		
	6271502	78311		

iii) Repeatability

a) Repeatability of injection

Standard drug solution was injected six times and its %RSD was calculated, **table 20.**

Table 20: Repeatability of injection

Concentration	Peak Area		%RSD	
	Metformin	Teneligliptin	Metformin	Teneligliptin
125 (µg/ml) Metformin 5 (µg/ml) Teneligliptin	6278397	79627	0.09	0.43
	6276164	79874		
	6279061	78968		
	6279365	79738		
	6266020	79736		
	6268003	79864		

5. Stability

The standard drug solutions were subjected to stability studies under room temperature and refrigerated conditions. Stability of solutions were analysed by looking for any changes in retention time, resolution, peak shape etc. The drug solutions were found to be stable for 5 hours at room temperature and 24 hours at refrigerated conditions, **fig. 46 and 47.**

Fig. 46: Stability of the analyte at room temperature

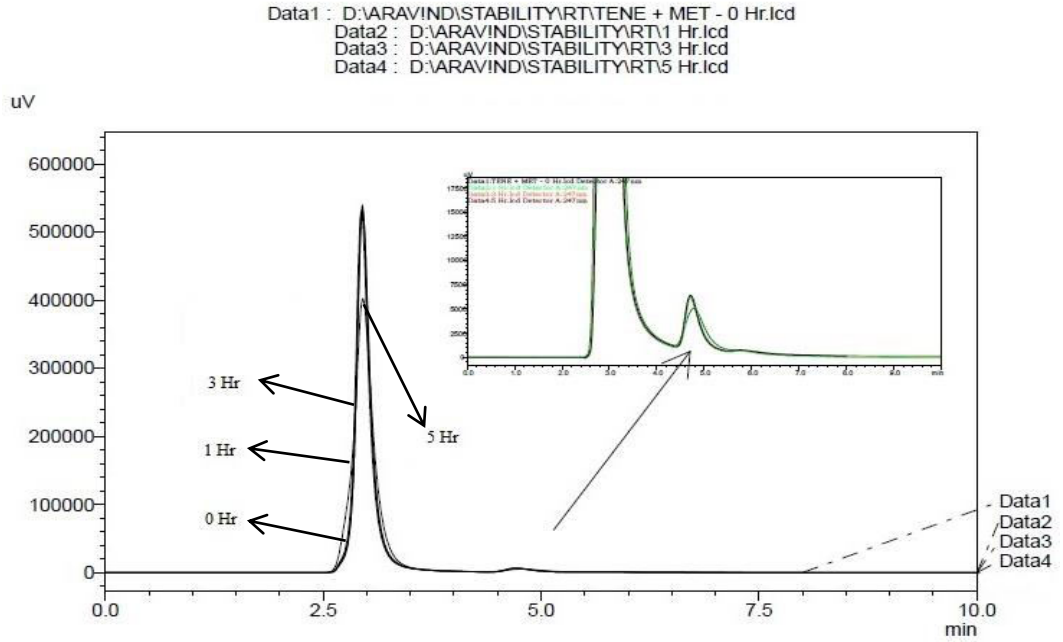
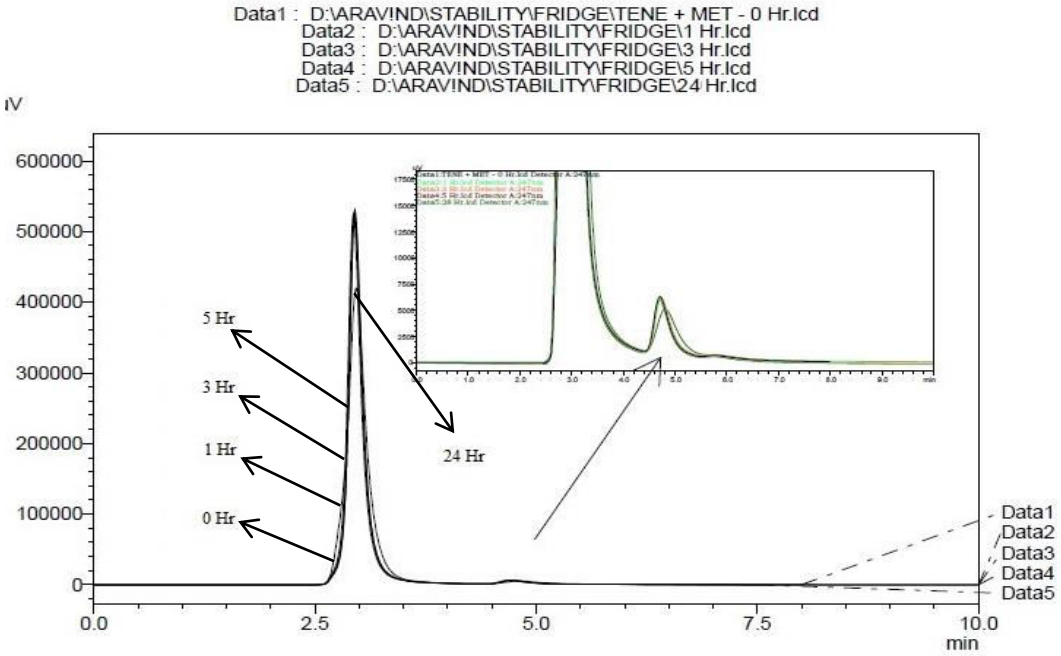


Fig. 47: Stability of the analyte at refrigerator



6. Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. Important parameters in the method were systemically varied and their effect on separation was measured as follows:-

⇔ ± 0.1 units of flow rate (**fig. 48**)

⇔ ± 2 units of mobile phase ratio (**fig. 49**)

⇔ ± 0.5 units of pH **(fig. 50**)

Fig. 48: Effect of flow rate

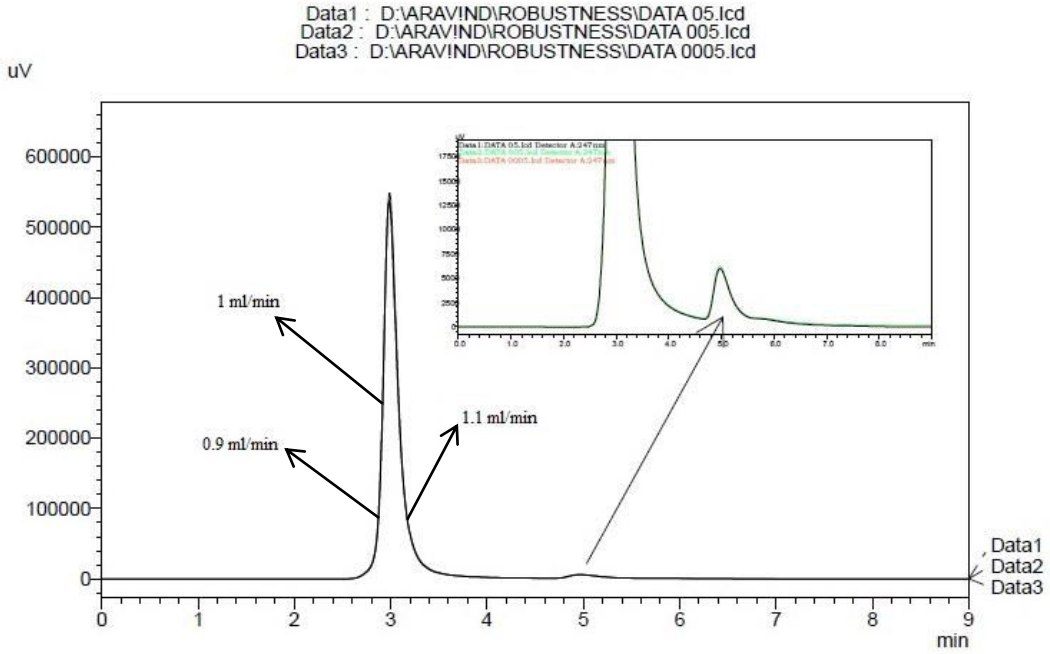


Fig. 49: Effect of mobile phase ratio

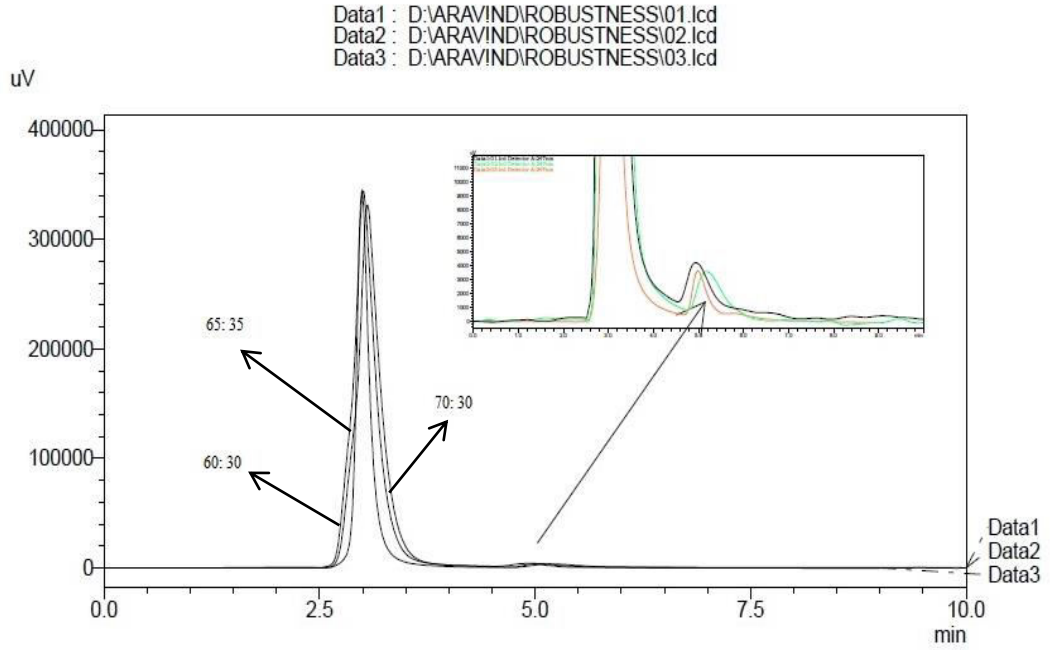
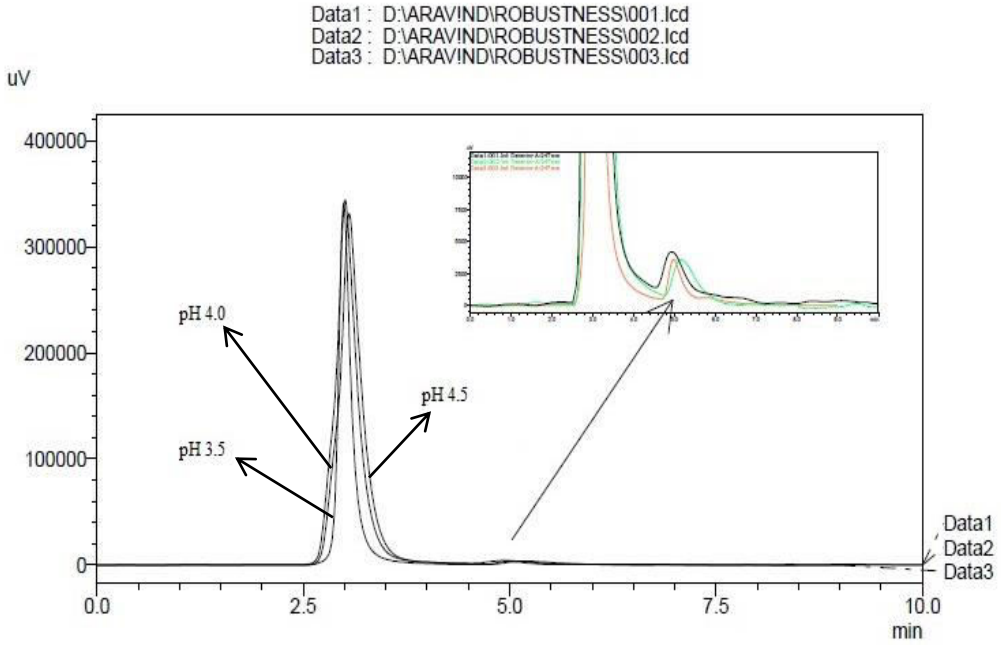


Fig. 50: Effect of pH



7. Specificity (forced degradation studies)

Sample degradation is also a technique for assessing specificity by deliberately degrading the sample and to look for the appearance of other peaks in the chromatogram. Here, the drugs were subjected to acid degradation (0.5 N HCL), base degradation (0.5 N NaOH), oxidative degradation (3% H_2O_2) and neutral conditions to achieve 10 to 20% degradation from the initial material.

Procedure

The stress testing was conducted as per ICH guidelines.

Forced degradation for the drugs were carried out under acid hydrolysis, base hydrolysis, oxidative stress conditions and neutral conditions.

Drug at concentration 1 mg/ml was used in all degradation studies.

In each study, blank and control (zero hour sample) were used to compare and calculate the % degradation.

There were four samples prepared in each stress test

1. Blank solution stored under normal condition
2. Blank solution subjected to stress like the drug
3. Zero time sample containing the drug which is stored under normal condition (control) and
4. Drug solution subjected to stress

i. Hydrolytic studies

a. Acidic condition

The solution was prepared by dissolving the drugs in methanol and the drugs were subjected to accelerated degradation under acidic condition with 0.5 N HCL and the sampling was done at every 15 minutes till sufficient degradation was achieved, **fig. 51 – 53.**

Fig. 51: Chromatogram of drug solution in acidic condition

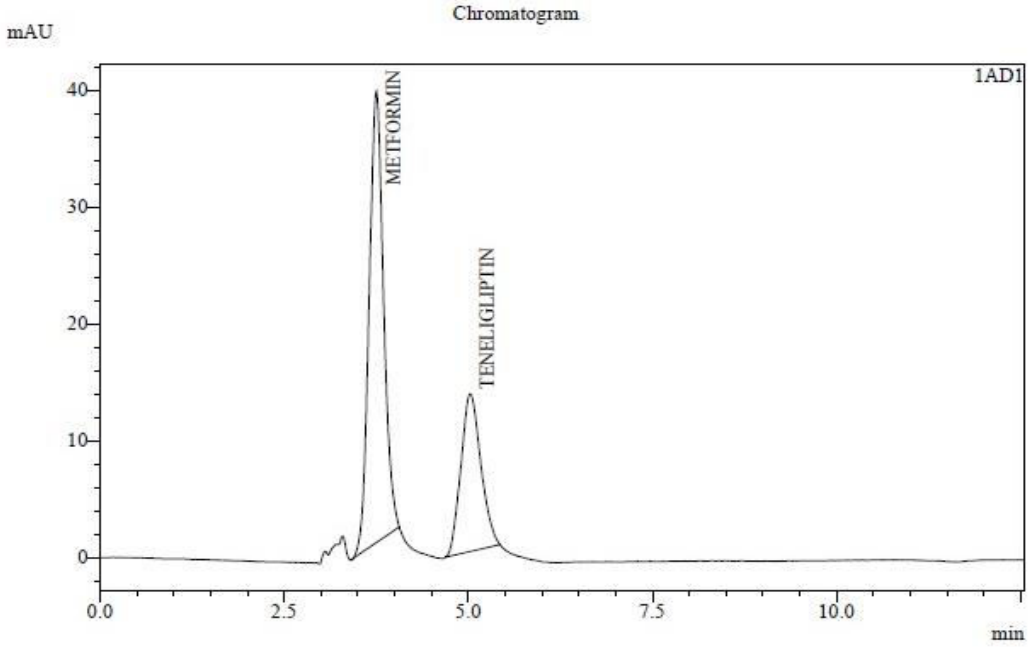


Fig. 52: Peak purity view of metformin

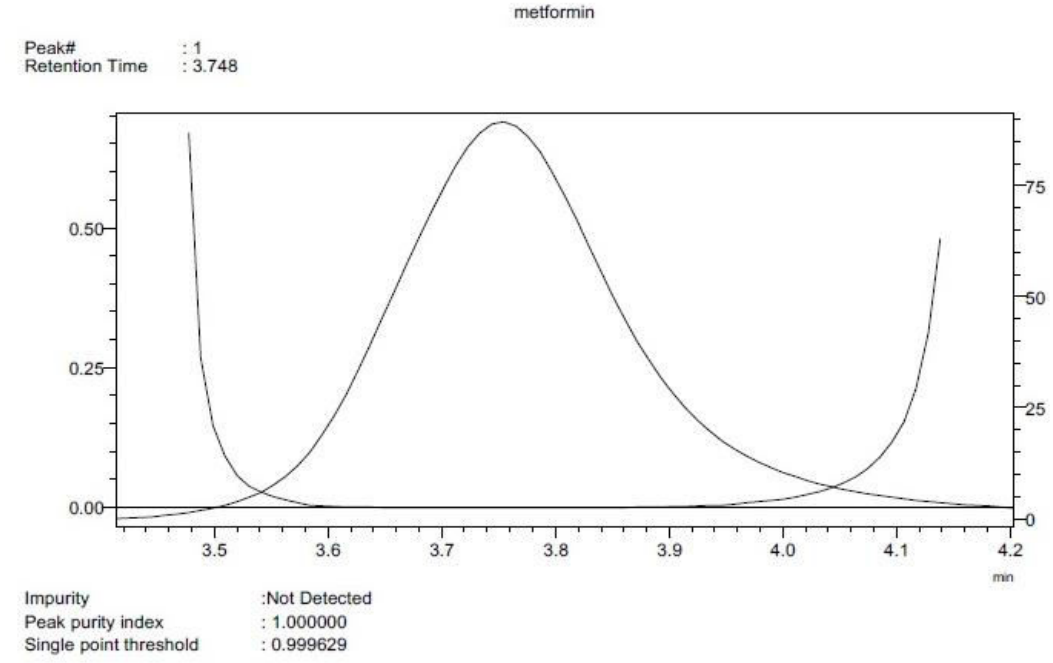
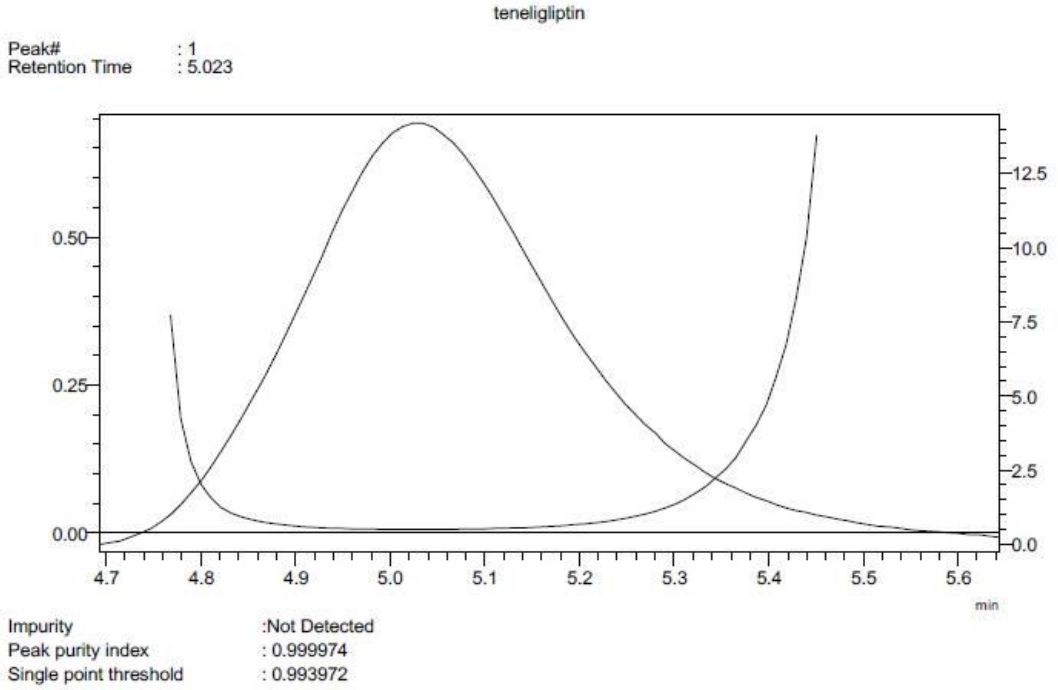


Fig. 53: Peak purity view of teneligliptin



b. ***Alkaline condition***

The drugs were dissolved in methanol and the drug was subjected to accelerated degradation under alkaline condition with 0.5 N NaOH and the sampling was done at every 15 minutes till sufficient degradation was achieved. The resulting solution was neutralized, appropriately diluted and chromatograms were recorded, **fig. 54 – 56.**

Fig. 54: Chromatogram of drug solution in alkaline condition

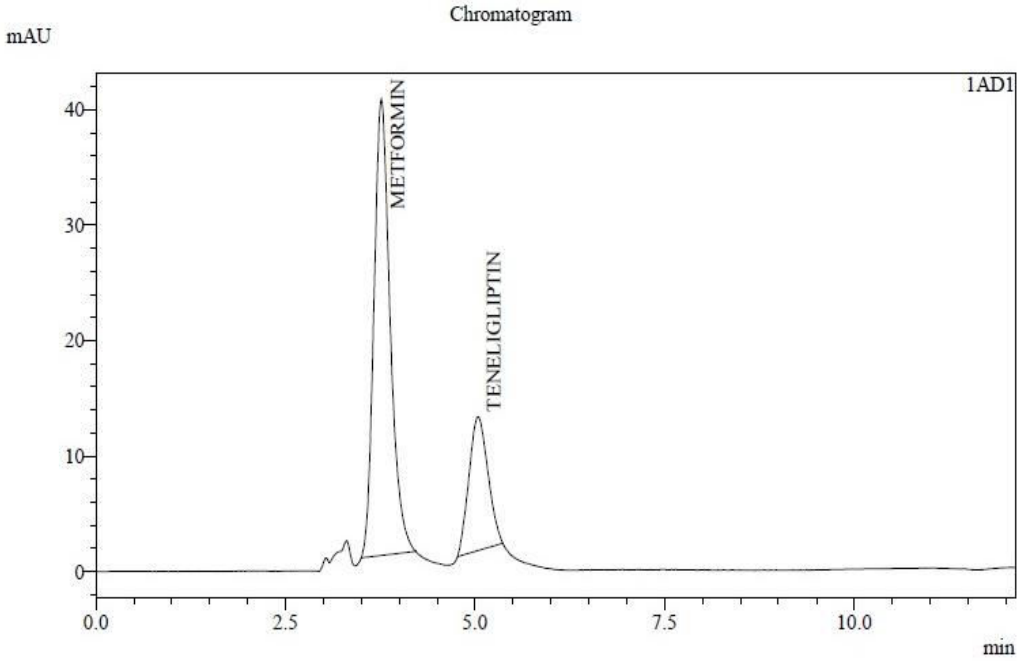


Fig. 55: Peak purity view of metformin

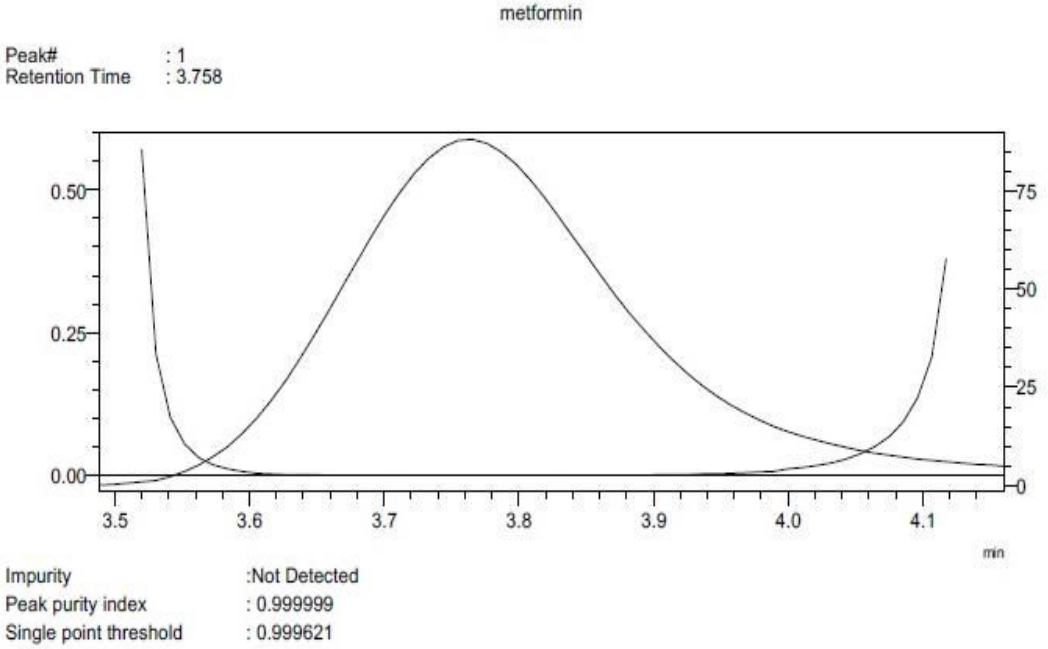
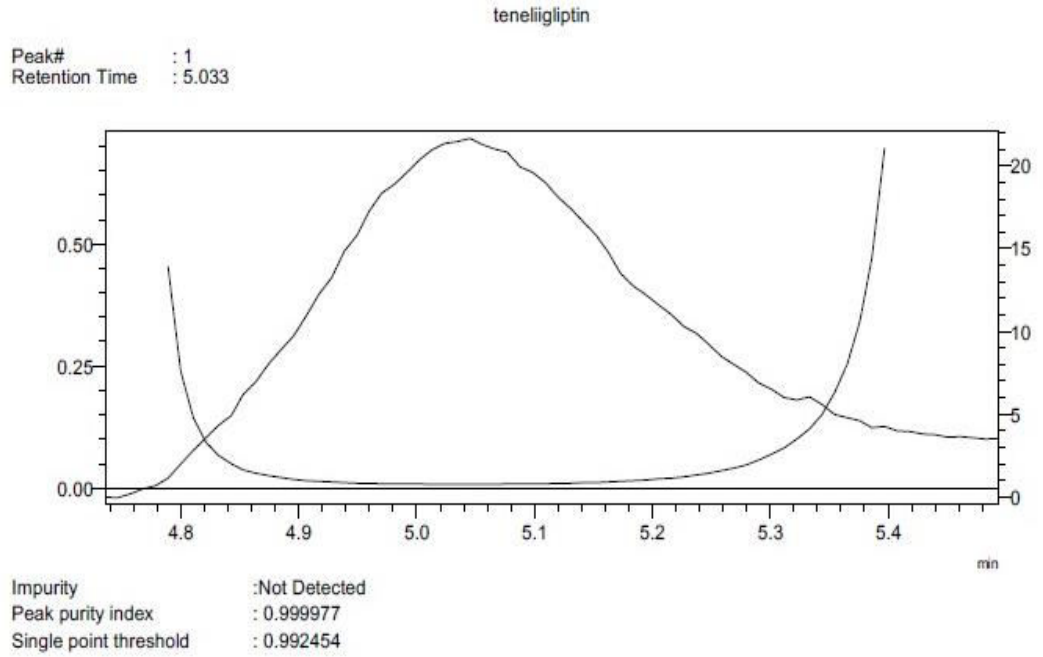


Fig. 56: Peak purity view of teneligliptin



c. Oxidative studies

Oxidation studies were performed in 3% H_2O_2 at room temperature. The resulting solution was appropriately diluted and chromatograms were recorded, **fig. 57 – 59.**

Fig. 57: Chromatogram of drug solution in oxidative condition

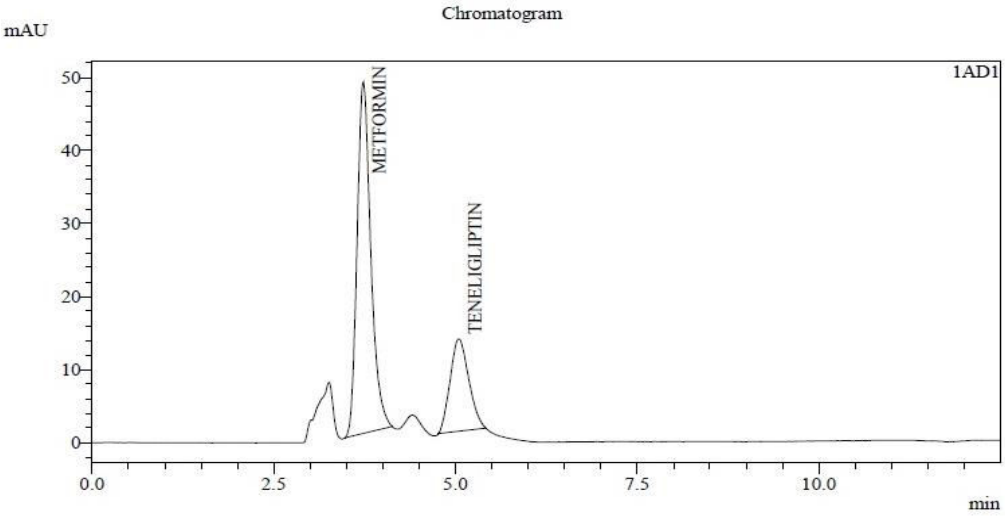


Fig. 58: Peak purity view of metformin

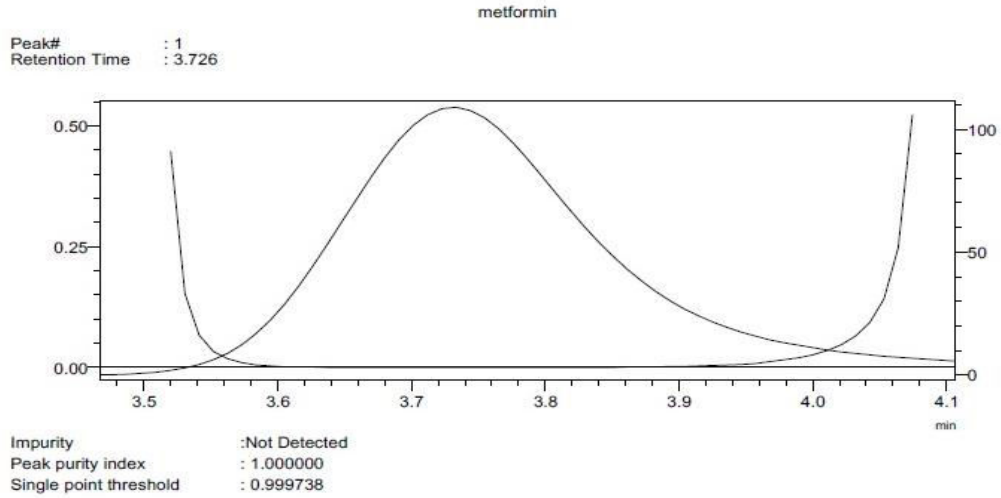
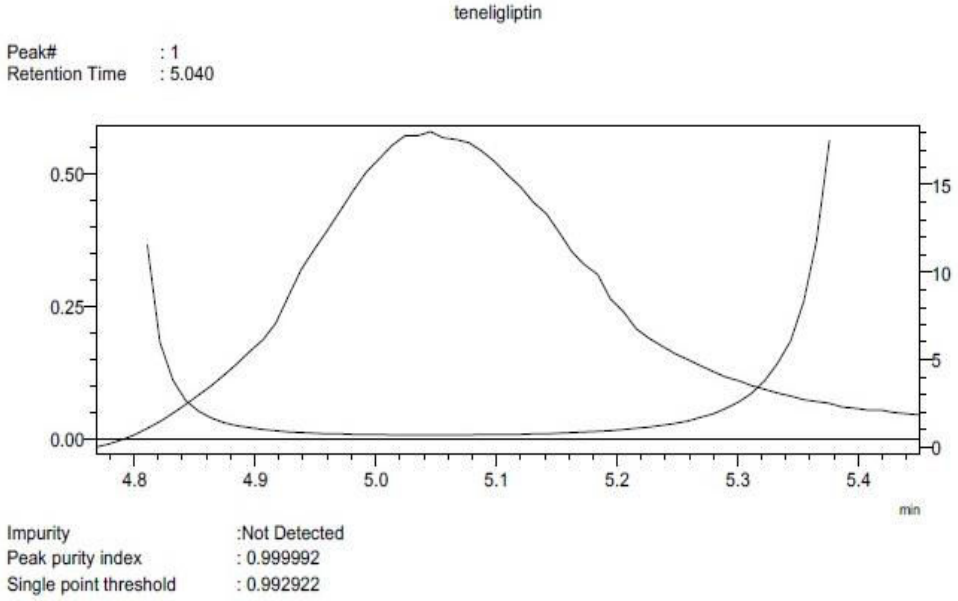


Fig. 59: Peak purity view of teneligliptin



d. Neutral condition

The drugs were dissolved in methanol and the solution was stored at room temperature and analysed periodically, **fig. 60 – 62.**

Fig. 60: Chromatogram of drug solution in neutral condition

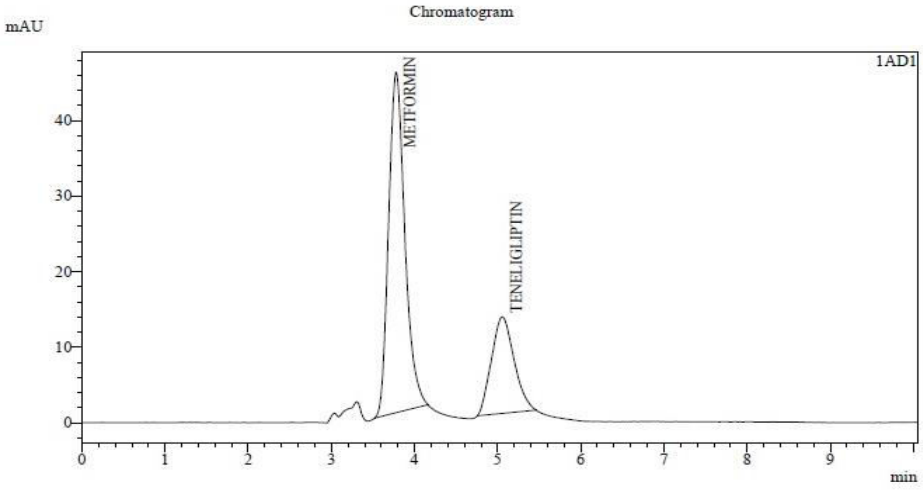


Fig. 61: Peak purity view of metformin

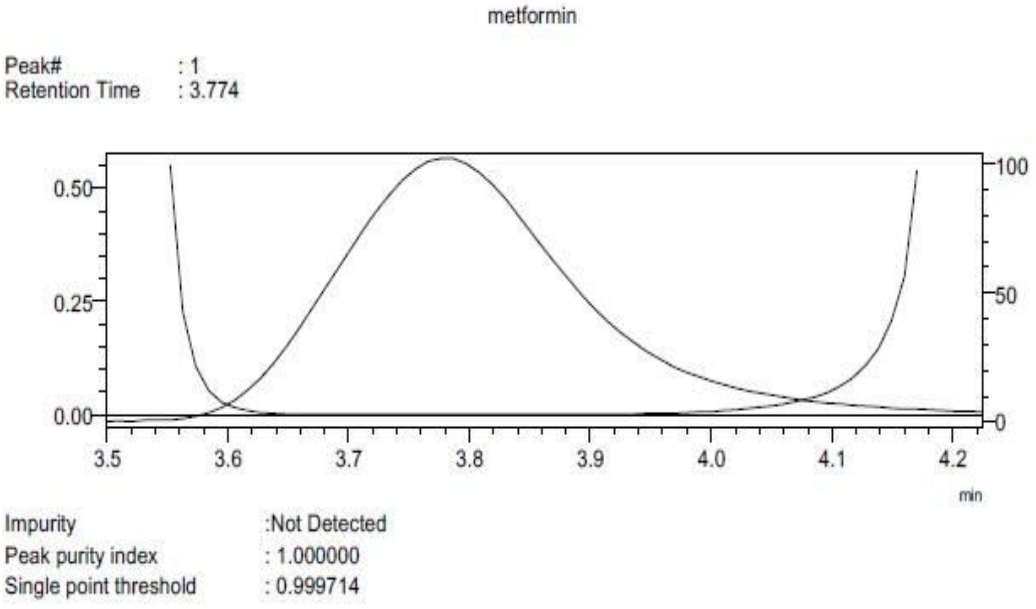
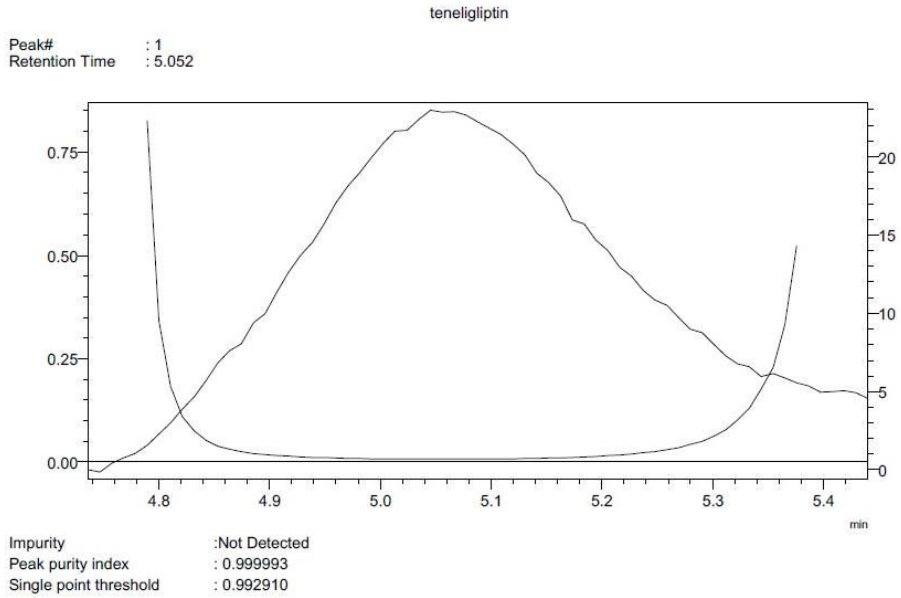


Fig. 62: Peak purity view of teneligliptin



In all degradation studies, there was no corresponding formation of degradation products except oxidation studies when compared to the standard solution of the drug. Photo diode array detection was used as an evidence of the specificity of the method and to evaluate the homogeneity of the drug peak. Chromatographic peak purity data was obtained from the spectral analysis report and peak purity index values close to 1 indicates homogenous peaks thus establishing the specificity of the assay method.

8. System suitability parameters

The USP defines parameters that can be used to determine system suitability prior to analysis. These parameters include plate number (N), tailing factor (T_f), capacity factor (k`), resolution (R_s) and relative standard deviation of peak area for repetitive injections, **table 21.**

Table 21: System suitability studies

Drug name	Number of theoretical plates (N)	Tailing Factor (T_f)	Capacity factor (k`)	Resolution (Rs)	Relative standard deviation of peak area (%RSD)
METFORMIN	1652	1.3	0.657	4.746	0.09
TENELIGLIPTIN	1381	1.5			0.43

ANALYSIS OF FORMULATION

1. Preparation of stock solution

A stock solution of metformin (2500 µg/ml) and teneligliptin (100 µg/ml) was prepared in methanol.

2. Preparation of sample solution

Ten tablets each containing 500 mg of metformin and 20 mg of teneligliptin were taken for the studies and the average weight was determined. Amount of powder equivalent to 125 mg of metformin and 5 mg of teneligliptin was taken and transferred to a 50 ml volumetric flask, and 25 ml methanol was added. The contents of the flask were shaken for 10 minutes, followed by dilution to volume with methanol to provide a solution containing 2500 µg/ml of metformin and 100 µg/ml of teneligliptin. This solution was filtered through a 0.45 mm membrane filter before injection.

3. Recording the chromatogram

A steady baseline was recorded with the fixed chromatographic conditions and 20 µL of standard drug solutions and sample solutions were injected and chromatograms were recorded, **fig. 63-72**. Calibration curve was plotted using the standard drug peak area versus concentration of standard solutions. The results of formulation analysis are given in, **table 22.**

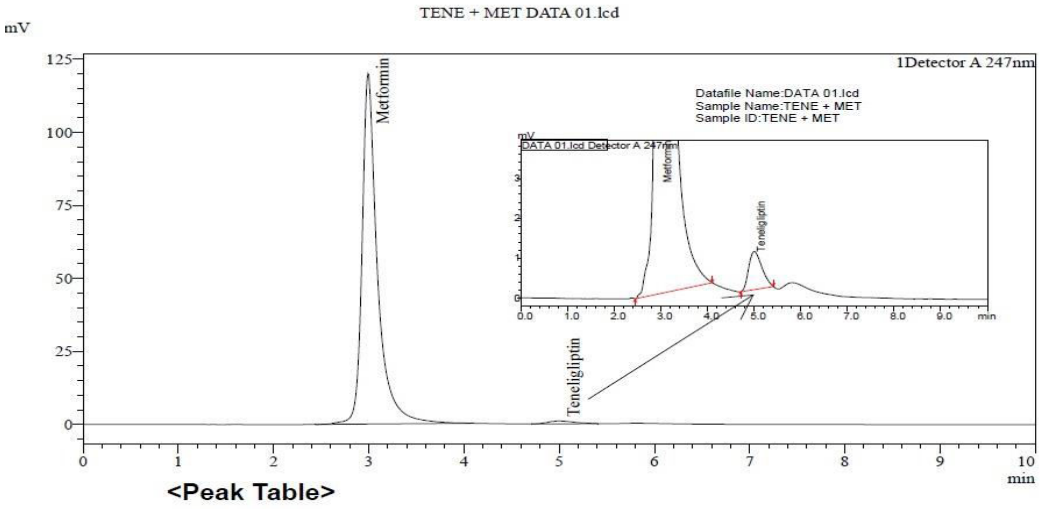
Table 22: Analysis of formulation

Drug	Amount of drug (mg/tablet)		% Label claim	%RSD*
	Labelled	Found		
Zita met plus (Teneligliptin 20 mg & Metformin 500mg)	20 mg	19.71 mg	98.55	1.42
	500 mg	483.33 mg	96.66	1.14

***RSD of 6 observations**

STANDARD CHROMATOGRAMS

Fig. 63: Chromatogram of standard 1 (MET 25µg/ml; TENE 1 µg/ml)

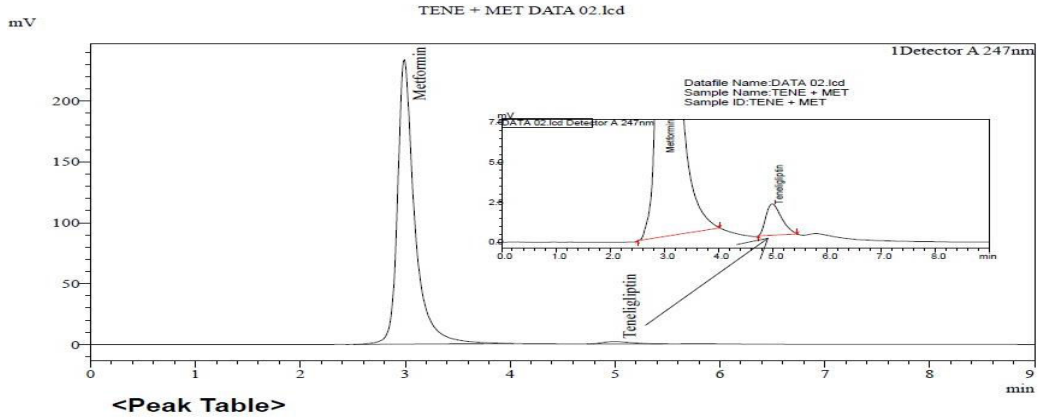


<Peak Table>

Detector A 247nm

Name	Ret. Time	Area
Metformin	2.962	1354287
Teneligliptin	4.923	15169
		1369456

Fig. 64: Chromatogram of standard 2 (MET 50µg/ml; TENE 2 µg/ml)

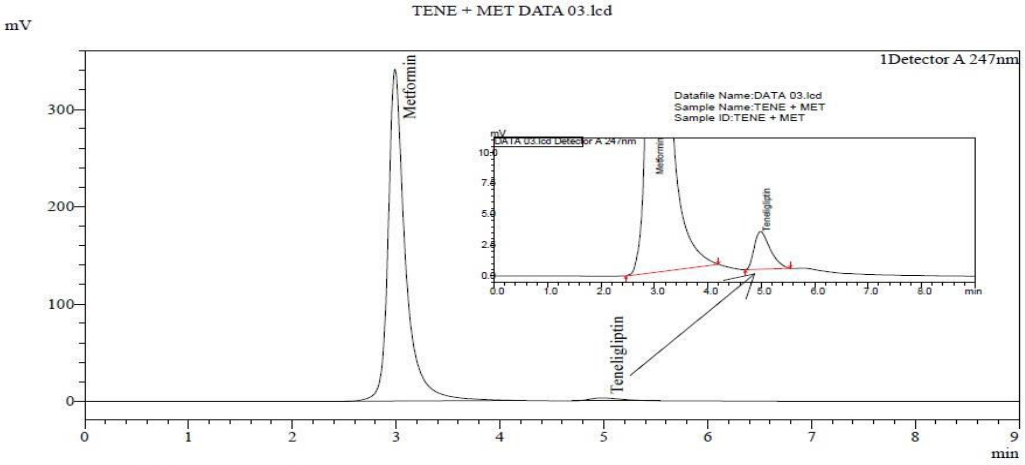


<Peak Table>

Detector A 247nm

Name	Ret. Time	Area
Metformin	2.959	2611634
Teneligliptin	4.914	29688
		2641322

Fig. 65: Chromatogram of standard 3 (MET 75µg/ml; TENE 3 µg/ml)

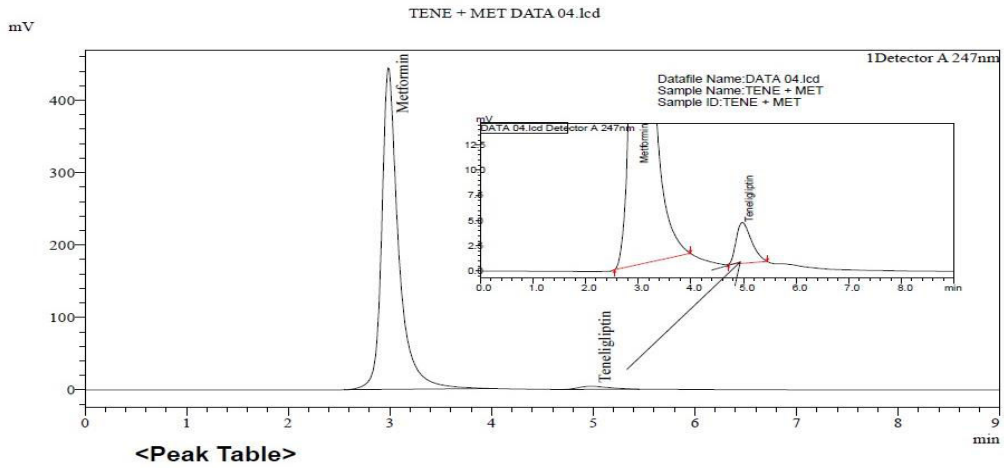


<Peak Table>

Detector A 247nm

Name	Ret. Time	Area
Metformin	2.959	3796175
Teneligliptin	4.905	46856
		3843031

Fig. 66: Chromatogram of standard 4 (MET 100µg/ml; TENE 4 µg/ml)

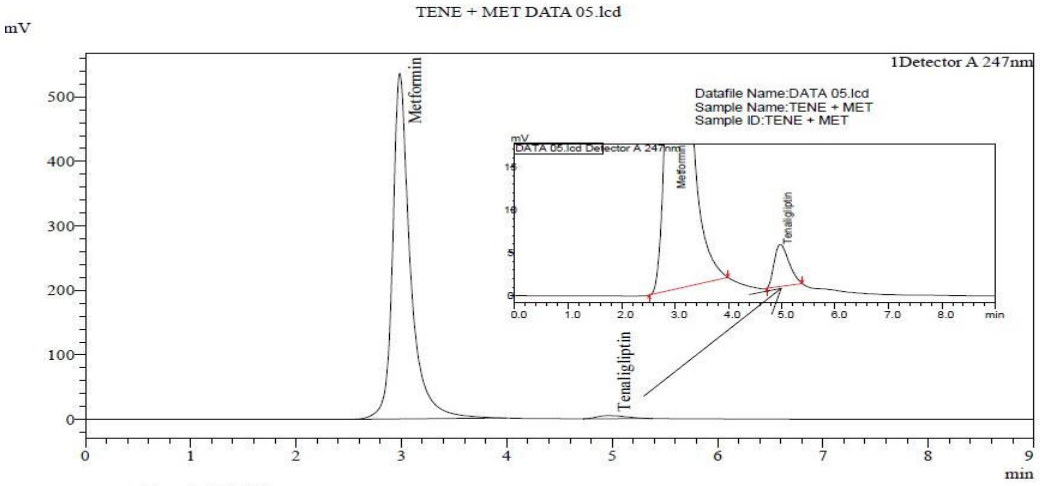


<Peak Table>

Detector A 247nm

Name	Ret. Time	Area
Metformin	2.952	4972965
Teneligliptin	4.892	62792
		5035757

Fig. 67: Chromatogram of standard 5 (MET 125µg/ml; TENE 5 µg/ml)

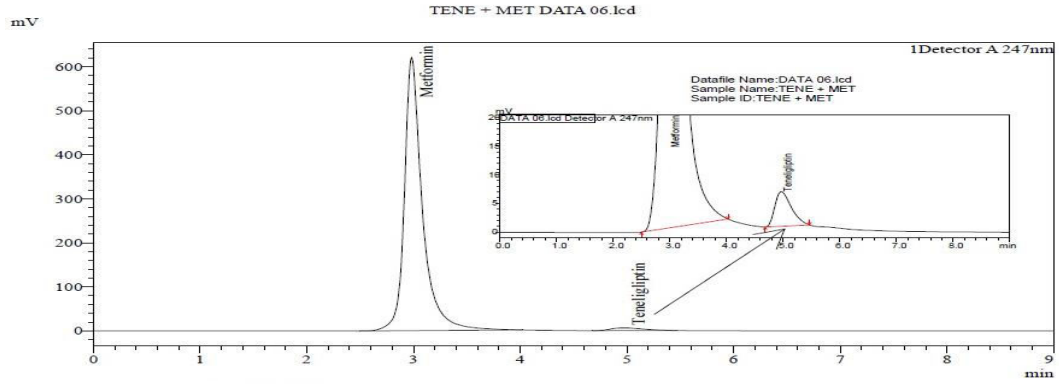


<Peak Table>

Detector A 247nm

Name	Ret. Time	Area
Metformin	2.951	6188947
Teneligliptin	4.892	79957
		6268904

Fig. 68: Chromatogram of standard 6 (MET 150µg/ml; TENE 6 µg/ml)

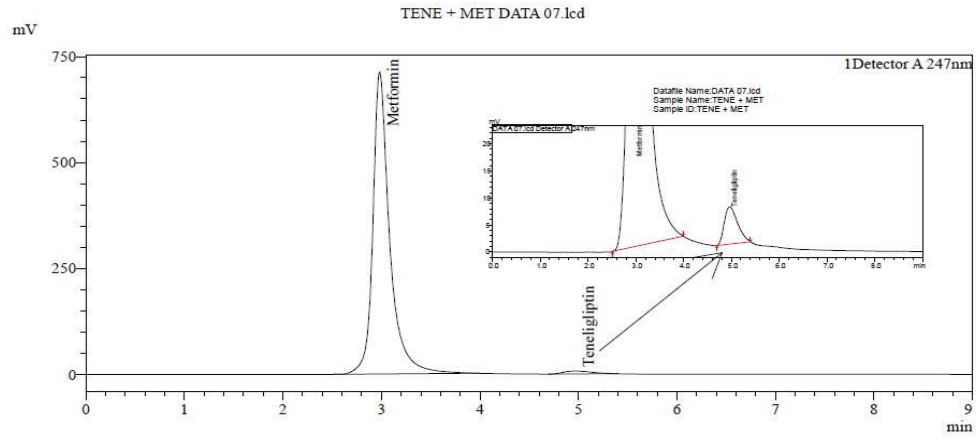


<Peak Table>

Detector A 247nm

Name	Ret. Time	Area
Metformin	2.949	7408120
Teneligliptin	4.892	98550
		7506670

Fig. 69: Chromatogram of standard 7 (MET 175µg/ml; TENE 7 µg/ml)

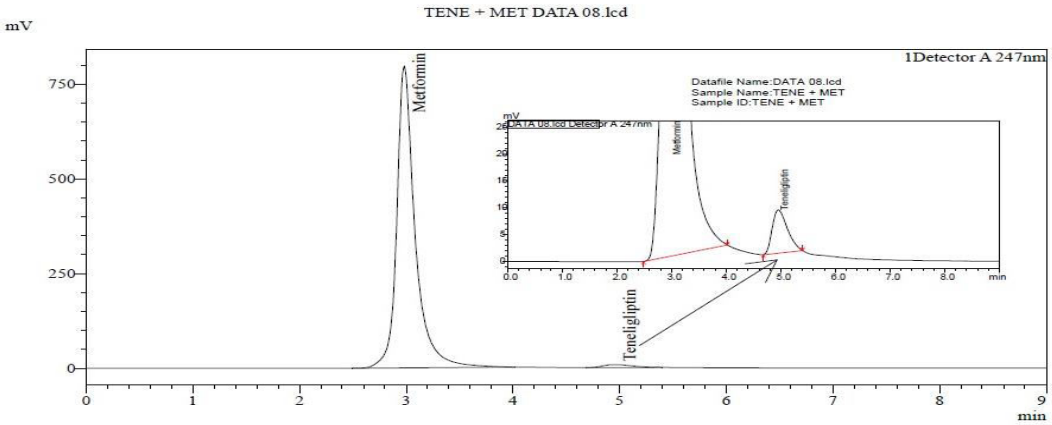


<Peak Table>

Detector A 247nm

Name	Ret. Time	Area
Metformin	2.949	8428047
Teneligliptin	4.893	112943
		8540990

Fig. 70: Chromatogram of standard 8 (MET 200µg/ml; TENE 8 µg/ml)

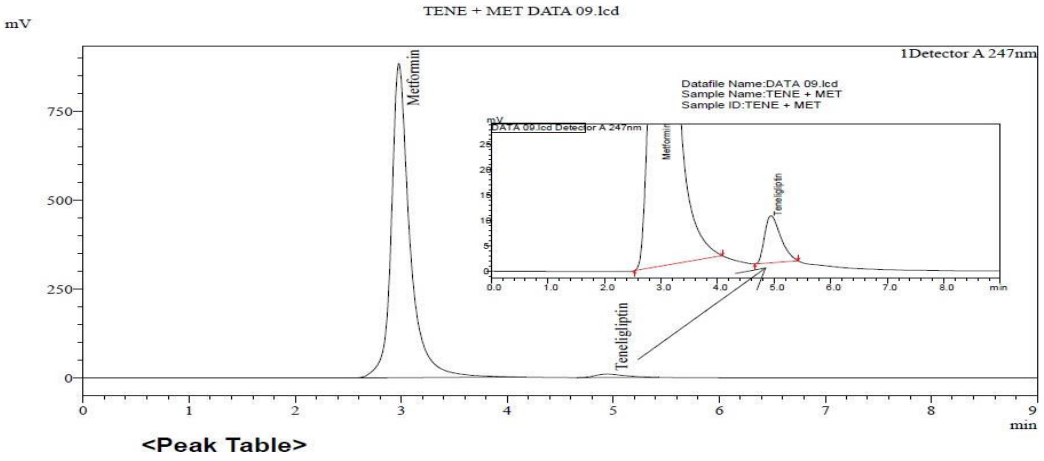


<Peak Table>

Detector A 247nm

Name	Ret. Time	Area
Metformin	2.947	9700838
Teneligliptin	4.892	131102
		9831940

Fig. 71: Chromatogram of standard 9 (MET 225µg/ml; TENE 9 µg/ml)

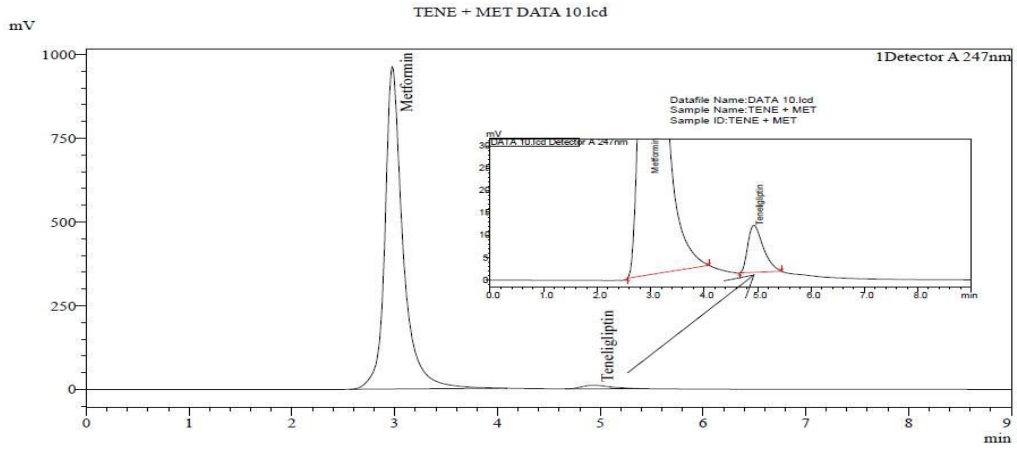


<Peak Table>

Detector A 247nm

Name	Ret. Time	Area
Metformin	2.946	10771794
Teneligliptin	4.889	147915
		10919709

Fig. 72: Chromatogram of standard 10 (MET 250µg/ml; TENE 10 µg/ml)



<Peak Table>

Detector A 247nm

Name	Ret. Time	Area
Metformin	2.944	11866708
Teneligliptin	4.889	157671
		12024379

7. SUMMARY AND CONCLUSION

Literature survey reveals no RP-HPLC and HPTLC methods for the simultaneous determination of metformin and teneligliptin in pharmaceutical dosage form. An attempt was made to develop validated methods like RP-HPLC and HPTLC for the simultaneous analysis of metformin and teneligliptin in combined dosage form.

7.1. HPTLC METHOD

For the determination of metformin and teneligliptin by HPTLC method different mobile phase systems were tried. It was found that a system comprising of methanol: tetrahydrofuran: ammonia (7: 3: 0.1, v/v/v) gave good separation with symmetric peaks, (R_f value of 0.29 ± 0.02 for metformin and 0.71 ± 0.02 for teneligliptin) at the selected wavelength of 258 nm. The method was validated as per ICH guidelines. Calibration curves were plotted with peak areas of standard drug versus concentration.

- Metformin was found to be linear in the concentration range of 6.25 to 17.5 μg/band.
- Teneligliptin was found to be linear in the concentration range of 0.25 to 7 μg/band.

The LOD values for metformin and teneligliptin were found to be 0.01 and 0.1μg/band, and their LOQ values were found to be 0.07 and 0.4μg/band respectively.

%RSD values for precision studies were found to be less than 2 which shows that the method is precise.

Recovery studies were carried out at 80%, 100% and 120% levels. Good recovery values show that the method is free from interferences. This method was successfully used for the simultaneous determination of metformin and teneligliptin from pharmaceutical dosage form.

Peak purity studies of the drugs (peak purity index values close to 1) showed that no impurities or degradation products eluted with drug peaks.

7.2. RP-HPLC METHOD

In RP-HPLC method, optimizations of different chromatographic parameters like selection of chromatographic method, detection wavelength, selection of mobile phase, mobile phase ratio, etc., were done. A wavelength of 247 nm was selected for the study. It was found that a system comprising of 10 mM potassium dihydrogen orthophosphate: methanol (35: 65, v/v) gave good resolution and peak characteristics, (retention times of metformin = 3 minutes and teneligliptin = 4.6 minutes). The method was validated as per ICH guidelines. Calibration curves were plotted with concentration versus peak area.

- Metformin was found to be linear in the concentration range of 25 to 250 μg/ml.
- Teneligliptin was found to be linear in the concentration range of 1 to 10 μg/ml.

The LOD values for metformin and teneligliptin were found to be 5 and 10 ng/ml, and their LOQ values were found to be 10 and 70 ng/ml respectively.

Stability studies were carried and the drug solutions were found to be stable for 5 hours and 24 hours at room temperature and refrigerated conditions, respectively.

Recovery studies were carried out at 80%, 100% and 120% levels. Good recovery values show that the method is free from interferences.

System suitability parameters like plate number (N), tailing factor (T_f), capacity factor (k`), resolution (R_s) and relative standard deviation of peak area for repetitive injections were studied and it was found that the values were within the limits.

Specificity of the method was studied by degrading the drugs in acidic, basic, oxidative and neutral conditions. Peak purity studies of the drugs (peak purity index values close to 1) showed that no impurities or degradation products eluted with drug peaks when compared to the standards. This indicates that the developed method is specific for the simultaneous determination of the drugs.

Summary of the method development studies is given in the **table 22.**

RP-HPLC and HPTLC methods were found to be simple, precise, specific and accurate. The developed methods were successfully validated according to ICH guidelines and hence these methods can be used for the simultaneous determination of metformin and teneligliptin from pharmaceutical dosage forms.

Table 22: Comparison of developed methods (HPTLC and RP-HPLC)

PARAMETERS	HPTLC		RP-HPLC	
	METFORMIN	TENELIGLIPTIN	METFORMIN	TENELIGLIPTIN
Linearity (Concentrations)	6.25 – 17.5 µg/band	0.25 – 0.7 µg/band	25 – 250 µg/ml	1 – 10 µg/ml
Correlation coefficient (r^2)	0.9991	0.9981	0.9991	0.9998
Accuracy (Recovery)				
80%	99.62	99.99	99.9	99.43
100%	101.25	100.98	99.78	100.27
120%	100.92	101.06	103.81	99.66
% RSD				
Repeatability	0.13	0.7	0.09	0.43
Intraday precision	0.32	1.5	0.71	0.37
Inter day precision	0.39	1.29	0.57	0.55
LOD	0.07 µg/band	0.1 µg/band	5 ng/ml	10 ng/ml
LOQ	0.1 µg/band	0.4 µg/band	10 ng/ml	70 ng/ml

7.3. STATISTICAL EVALUATION[19, 20]

The above developed analytical methods were statistically compared by *student t* test using Graph Pad Instat software. This results shows that there is no significant difference among these methods. Hence, the developed methods can be used for routine analysis of metformin and teneligliptin from pharmaceutical dosage form, **table 23.**

Table 23: Statistical comparisons between developed methods

Methods	Metformin	Teneligliptin
RP-HPLC vs HPTLC	t_c = 0.3937 P value = 0.0942	t_c = 12.626 P value = 0.4846

t_c = calculated 't' value; t_t = table 't' values (t_t = 2.228 for n = 6)
Hypothesis (H_o): no statistical difference exists between two methods. $t_c < t_t$: H_o Hypothesis is accepted (P > 0.05)
The two-tailed P value was considered no significant.

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